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## CONTENTS

### SEC. C.—BOTANICAL SCIENCES

	Page
The Nitrate Reduction Test and its Significance in the Detection of <i>Bacillus Larvae</i> — <i>A. G. Lochhead</i> - - - - -	79
Comparative Studies in Potato Virus Diseases— <i>Donald F. Putnam</i> - - - - -	87
The Parasitism of <i>Cladosporium fulvum</i> Cooke and the Genetics of Resistance to it— <i>Arthur N. Langford</i> - - - - -	108

### SEC. D.—ZOOLOGICAL SCIENCES

Studies on the Genus <i>Rhagoletis</i> (Tryptidae) with Special Reference to <i>Rhagoletis pomonella</i> (Walsh)— <i>A. D. Pickett</i>	53
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# Canadian Journal of Research

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VOL. 15, SEC. C.

MARCH, 1937

NUMBER 3

## THE NITRATE REDUCTION TEST AND ITS SIGNIFICANCE IN THE DETECTION OF *BACILLUS LARVAE*<sup>1</sup>

By A. G. LOCHHEAD<sup>2</sup>

### Abstract

*Bacillus larvae* differs from most nitrate-reducing species in its ability to accumulate nitrite in nutrient solutions containing but small amounts of nitrate (0.001%). Most nitrate-reducing organisms show no accumulation of nitrite at this concentration owing to assimilation of nitrate or disappearance of nitrite through reduction or assimilation. With many nitrate-reducing bacteria disappearance of nitrite keeps pace with nitrite formation only up to a certain concentration, varying with the organism, above which nitrite may accumulate.

The ability of *B. larvae* to accumulate nitrite in semi-solid carrot or turnip extract media with no added nitrate is of considerable aid in the cultural test for this organism. Of five other organisms concerned with brood disease or occurring as contaminants in comb, which were grown in association with *B. larvae*, none showed interference with accumulation of nitrite by the latter except *B. orpheus*. With this species a positive nitrite test was dependent on the relative development of the organisms, *B. larvae* exerting a certain antagonistic action. None of the eight species of bacteria tested prevented recognition of growth of *B. larvae* in the semi-solid medium.

### Introduction

The application of the nitrite test has been found to be of considerable diagnostic value in the detection of *Bacillus larvae*, the organism causing American foulbrood of bees. As reported previously by the writer (4), this organism, grown in a suitable medium containing carrot extract, and without added nitrate, gives a positive reaction for nitrite. Under similar conditions all other spore-forming organisms so far tested, as well as many miscellaneous types that may occur in foulbrood diseases or as common contaminants, have not given a similar positive test.

Inclusion of the nitrite test in the routine cultural control for viable spores of *B. larvae* has confirmed its usefulness. Sturtevant (6) likewise found it a fairly delicate and reliable indicator of vegetative growth of this organism, while recently Hitchcock (3) reports successful application of the test. Even in the absence of visible growth, a positive nitrite reaction may be regarded as presumptive evidence of the presence of viable cells of *B. larvae*.

The experiments here described were undertaken to study more closely:—

(i) The relation of *B. larvae* and other organisms to nitrate reduction and nitrite accumulation.

(ii) The possible influence of contaminants in affecting the recognition of *B. larvae* and its characteristic nitrite reaction.

<sup>1</sup> Manuscript received November 28, 1936.

Contribution No. 41 (Journal Series) from Division of Bacteriology, Dominion Experimental Farms, Ottawa.

<sup>2</sup> Dominion Agricultural Bacteriologist.

### Experimental

#### (1) Effect of Different Concentration of Nitrate on the Nitrite Reaction

A study was made of the effect of additions of potassium nitrate in varying concentration to media inoculated with *B. larvae* and a number of stock strains of organisms which showed nitrate reduction in standard broth containing 0.1% potassium nitrate. Using two basic solutions of yeast-peptone-carrot broth and beef-peptone broth, media were prepared containing 0.1%, 0.01% and 0.001% potassium nitrate and inoculated respectively with three strains of *B. larvae* and six other organisms known to be nitrate-reducing species. Yeast-peptone-carrot media were included in order to provide a series supporting good growth of *B. larvae*, an organism which does not develop in standard nutrient broth. This basic medium is prepared by adding to 1 litre water, 3 gm. yeast extract (Difco), 10 gm. peptone (Difco), 0.5 gm. di-potassium hydrogen phosphate ( $K_2HPO_4$ ) and 200 ml. of clear carrot extract prepared by mincing 100 gm. carrots, grinding with 250 ml. distilled water and filtering (5).

The results of the test for nitrite, as well as for nitrate in cases where the former was negative, are shown in Table I. It will be seen that *B. larvae*, provided the medium is suitable for growth, gives a positive test for nitrite irrespective of the concentration of potassium nitrate used, showing a positive test in the yeast-peptone-carrot medium without added nitrate, due probably

TABLE I  
NITRITE ACCUMULATION BY *B. larvae* AND VARIOUS NITRATE-REDUCING ORGANISMS IN DIFFERENT MEDIA

Organism	Yeast-peptone-carrot broth											
	+0.1% KNO <sub>3</sub>				+0.01% KNO <sub>3</sub>				+0.001% KNO <sub>3</sub>			
	Growth	NO <sub>2</sub>	NO <sub>3</sub>	Growth	NO <sub>2</sub>	NO <sub>3</sub>	Growth	NO <sub>2</sub>	NO <sub>3</sub>	Growth	NO <sub>2</sub>	NO <sub>3</sub>
<i>B. larvae</i> (Str. 1)	+	+		+	+		+	+		+	+	
<i>B. larvae</i> (Str. 2)	++	++		++	++		++	++		++	++	
<i>B. larvae</i> (Str. 3)	++	++		++	++		++	++		++	++	
<i>B. cereus</i> (135)	+	+		+	-	-	+	-	-	+	-	-
<i>Bacillus</i> sp. (101)	++	++		++	-	-	++	-	-	++	-	-
<i>Esch. coli</i> (117)	++	++		++	-	-	++	-	-	++	-	-
<i>Salmonella</i> sp. (136)	++	++		++	-	-	++	-	-	++	-	-
<i>Eberl. typhi</i> (116)	++	++		++	-	-	++	-	-	++	-	-
<i>Micrococcus</i> sp. (169)	++	++		+	-	-	++	-	-	++	-	-
Culture	Beef-peptone broth											
	+0.1% KNO <sub>3</sub>				+0.01% KNO <sub>3</sub>				+0.001% KNO <sub>3</sub>			
	Growth	NO <sub>2</sub>	NO <sub>3</sub>	Growth	NO <sub>2</sub>	NO <sub>3</sub>	Growth	NO <sub>2</sub>	NO <sub>3</sub>	Growth	NO <sub>2</sub>	NO <sub>3</sub>
<i>B. larvae</i> (Str. 1)	-	-	+	-	-	+	-	-	-	sl.	-	-
<i>B. larvae</i> (Str. 2)	-	-	+	-	-	+	-	-	-	sl.	-	-
<i>B. larvae</i> (Str. 3)	-	-	+	-	-	+	-	-	-	sl.	-	-
<i>B. cereus</i> (135)	+	+		+	-	-	+	-	-	+	-	-
<i>Bacillus</i> sp. (101)	++	++		++	+	-	++	-	-	++	-	-
<i>Esch. coli</i> (117)	++	++		++	sl.	-	++	-	-	++	-	-
<i>Salmonella</i> sp. (136)	++	++		++	+	-	++	-	-	++	-	-
<i>Eberl. typhi</i> (116)	++	++		++	+	-	++	-	-	++	-	-
<i>Micrococcus</i> sp. (169)	++	++		+	-	-	++	-	-	++	-	-

to the presence of traces of nitrate derived from the carrot extract. On the other hand, the other organisms tested, though giving a nitrite test with media containing 0.1% potassium nitrate, failed for the most part with 0.01%, and gave entirely negative tests with 0.001% potassium nitrate. The negative tests for nitrate where no nitrite was found indicate that with the lower concentrations of nitrate, there has been either assimilation of nitrate or disappearance of nitrite, through further reduction or by assimilation. In the case of *B. larvae* neither reduction of nitrites nor assimilation of nitrite or nitrate is apparent.

#### (2) *The Nitrite Test as Affected by Varying Concentrations of Nitrate and Nitrite in the Medium*

To examine further the behavior of different organisms to the nitrite test, two series of solutions were prepared from standard nutrient broth (Difco), one series with seven concentrations of potassium nitrate, from 0.1% to 0.001%, the other with equivalent amounts of potassium nitrite, from 0.0842% to 0.00084%. Tubes in both series were inoculated uniformly with a loopful of suspension of the organism tested. In all, 42 organisms were studied, 40 of which were nitrate-reducing in standard 0.1% potassium nitrate solution, with two non-reducing species as controls. The organisms consisted of various stock strains together with miscellaneous types freshly isolated from soil, air, milk, etc., some of which were not specifically identified. All cultures were incubated at 37° C. and tested for nitrite after one, two and seven days by the  $\alpha$ -naphthylamine-sulphanilic acid test.

Of the 40 nitrate-reducing types studied only two gave positive nitrite tests with all concentrations of potassium nitrate. The remaining 38 species gave negative results in the lower concentrations to an extent which varied with the species. In Table II are shown results from typical members of this group (Group A) as well as from the two former (Group B) and the two non-reducing controls (Group C).

A comparison of the results for nitrate broth with those for nitrite broth of equivalent concentration shows general agreement in the limiting concentrations giving positive nitrite tests for the organisms of Group A. The results suggest that the negative nitrite tests with the lower concentrations of nitrate are due to further reduction or assimilation of nitrite, preventing any accumulation of the latter. This is confirmed by the frequent disappearance of nitrite, on prolonged incubation, from cultures which gave positive tests after one day. With some organisms nitrites were absent more promptly in the nitrite series than in cultures with equivalent concentrations of nitrate. This is understandable in view of a probable lag in the latter series as compared with that where nitrite is present originally.

Disappearance of nitrites apparently keeps pace with formation of nitrites only up to a certain concentration, varying with the organism, and above which nitrite may accumulate. The results indicate the importance of a sufficiently high concentration of nitrate in media used for the determination

TABLE II  
EFFECT OF VARYING CONCENTRATIONS OF NITRATE AND NITRITE ON THE NITRITE TEST

Group*	Test organism	Cult. No.	Nitrate broth—per cent $\text{KNO}_3$ —( $\text{NO}_2$ test after 1, 2, 7 days)							
			0.1%		0.05%		0.02%		0.01%	
			1	2	7	1	2	7	1	2
A	<i>B. subtilis</i>	246	+	+	+	+	+	+	—	—
	<i>B. cereus</i>	135	—	—	—	—	—	—	—	—
	<i>B. mycoides</i>	381	—	—	—	—	—	—	—	—
	<i>B. albolactis</i>	213	—	—	—	—	—	—	—	—
	<i>B. pseudolautanicus</i>	154	—	—	—	—	—	—	—	—
	<i>B. oryzens</i>	226	—	—	—	—	—	—	—	—
	<i>Esch. coli</i>	117	—	—	—	—	—	—	—	—
	<i>Aerob. diazot</i>	133	—	—	—	—	—	—	—	—
	<i>Salmonella</i> sp.	136	—	—	—	—	—	—	—	—
	<i>Eh. typhi</i>	251	—	—	—	—	—	—	—	—
B	<i>Staph. aureus</i>	292	—	—	—	—	—	—	—	—
	<i>Micrococcus</i> sp.	329	+	+	+	+	+	+	+	+
	<i>Flavobacterium</i> sp.	170	sl.	+	sl.	—	—	—	—	—
	<i>B. vulgaris</i>	389	—	—	—	—	—	—	—	—
C	<i>B. alvei</i>	127	—	—	—	—	—	—	—	—
	Control—not inoc.	—	—	—	—	—	—	—	—	—
D										

\*Group A—Nitrate reduced to nitrite, partial reduction or assimilation of nitrite.

Group B—Nitrate reduced to nitrite, no reduction or assimilation of nitrite.

Group C—No reduction of nitrate or nitrite.

TABLE II—*Concluded*  
EFFECT OF VARYING CONCENTRATIONS OF NITRATE AND NITRITE ON THE NITRITE TEST

of nitrate reduction by bacteria, particularly since positive results are significant, while negative results must be supplemented by further tests before an organism can be designated as a non-reducer. This point has been emphasized by Bronfenbrenner and Schlesinger (1), ZoBell (7) and particularly by Conn (2) who points out the need for caution in designating species as non-nitrate reducing. Use of a solution of too low concentration (e.g., 0.01 to 0.02% potassium nitrate) has doubtless been responsible for the classification, as non-reducing species, of organisms that may be nitrate-reducing, and has contributed to much of the confusion in the literature as to this characteristic.

The two species in Group B (Table II), a *Micrococcus* isolated from meat-curing pickle and a species of *Flavobacterium* isolated from the surface of a cow's udder, and causingropy milk, represent less common types. These organisms reduce nitrates without causing disappearance of nitrite through further reduction or assimilation. Moreover the accumulation of nitrite in a medium containing but 0.001% potassium nitrate also suggests that none of the original nitrate is assimilated for cell building. In these respects the organisms appear to behave like *B. larvae*.

With the species in Group C no reduction is indicated, the persistence of nitrite in the medium with the lowest concentration of potassium nitrite indicating that the failure to show nitrite in the nitrate series is due to absence of reduction rather than to disappearance of nitrite.

### (3) Effect of Contaminants on Recognition of *B. larvae* and the Nitrite Test

In the cultivation of *Bacillus larvae* from combs suspected of containing American foulbrood, or for the detection of viable spores remaining after treatment of diseased comb with disinfectants, surprisingly little trouble due to growth of contaminating organisms has been encountered. Occasional contamination does occur, due in the majority of cases observed, to the presence of spore-forming organisms of the *mesentericus* or *vulgatus* type. The whole trend of the cultural tests, however, suggests a possible antagonistic action between *B. larvae* and other organisms.

To examine the effect of association of *B. larvae* with other bacteria, three strains of the former, isolated from different sources, were each tested in association with eight other species. Of the latter, three are concerned with bee disease (*B. orpheus*, *B. alvei* and *Str. apis*), two were isolated as contaminants from diseased comb (*B. vulgatus*, *Bacillus* sp.) while the remaining three were other common types (*B. subtilis*, *Esch. coli*, *Staph. aureus*).

Tests were made in yeast-peptone-turnip semi-solid medium. This is similar to the yeast-peptone-carrot medium previously described (4) except that turnip extract is used in place of carrot, having been found to support better growth of *B. larvae*, and now employed in the routine examination for this organism. In one series the strain of *B. larvae* examined and the test organism were inoculated simultaneously, while in a second series the test organism was added 48 hours after *B. larvae*. Cultures were all held at 37° C. After 48 hours, examination was made for *B. larvae* in the first series, and

TABLE III  
EFFECT OF ASSOCIATION OF *B. larvae* AND OTHER ORGANISMS

Organism	Nitrate broth, 0.1% KNO <sub>3</sub>		Nitrite broth, 0.002% KNO <sub>3</sub>		Yeast-peptone-turp semi-solid medium		Test organism inoculated 48 hr. after <i>B. larvae</i> and test org.	Test organism inoculated 48 hr. after <i>B. larvae</i>	Growth of <i>B. larvae</i>	Growth of test org.	NO <sub>3</sub>	Growth of test org.	NO <sub>2</sub>	Yeast-peptone- turnip agar, antagonistic effect by <i>B. larvae</i>	
	NO <sub>3</sub>	NO <sub>3</sub>	Inoculated simply	Growth	NO <sub>3</sub>	Growth of <i>B. larvae</i>									
<i>Controls</i>															
<i>B. larvae</i> (from worker scale)	No growth	No growth	+	+	-	-	-	-	-	-	-	-	-	-	None
<i>B. larvae</i> (from drone larva)	No growth	No growth	+	+	-	-	-	-	-	-	-	-	-	-	Definite
<i>B. larvae</i> (from queen cell)	No growth	No growth	+	+	-	-	-	-	-	-	-	-	-	-	None
<i>Test Organisms</i>															
<i>B. subtilis</i> (246)	+	++	-	+++	+	Slight	Good	Good	Good	Good	Good	+	+	+	None
<i>B. orpheus</i> (226)	++	++	-	++	+	Fair	Good	Good	Good	Good	Good	+	+	+	None
<i>Esch. coli</i> (117)	++	++	-	++	+	Slight	Good	Good	Good	Good	Good	+	+	+	None
<i>Staph. aureus</i> (292)	++	++	-	++	+	Slight	Good	Good	Good	Good	Good	+	+	+	Slight
<i>B. vulgaris</i> (389)	++	++	-	++	+	Fair	Good	Good	Good	Good	Good	+	+	+	None
<i>B. alfei</i> (127)	++	++	-	++	+	Slight	Good	Good	Good	Good	Good	+	+	+	None
<i>Bacillus</i> sp. (390)	++	++	-	++	+	Fair	Good	Good	Good	Good	Good	+	+	+	Slight
<i>Str. apis</i> (239)	++	++	-	++	+	Fair	Good	Good	Good	Good	Good	+	+	+	None

for comparative development of the test organism in the second series, the nitrite test being also made in all cases. In addition, cultures of *B. larvae* were made on yeast-peptone-turnip agar plates to which cultures of the test organisms were added, after 48 hours, to allow any visible antagonistic effect to be noted.

With each of the strains of *B. larvae* used results were similar, and these are summarized in Table III. In no case did simultaneous inoculation of the test organism prevent recognition of *B. larvae* microscopically, even though the contaminant developed well. Of the eight test organisms, four were nitrate-reducing and able to cause disappearance of 0.002% nitrite, and prevent accumulation of nitrite in the special medium when grown singly. Grown simultaneously with *B. larvae*, three prevented nitrite accumulation. The four other test organisms were non-nitrate reducing, and unable to reduce or assimilate small quantities of nitrite. They were unable to prevent growth of *B. larvae* or prevent accumulation of nitrite by the latter when grown in association.

Where the test organism was inoculated 48 hours after *B. larvae*, rather less development of the contaminant occurred, with the exception of *B. subtilis* and *Esch. coli*. After 48 hours' further incubation, nitrite accumulation by *B. larvae* was not detectable in the case of these two organisms only. With *B. orpheus* the positive nitrite test suggested an inhibiting action of *B. larvae*. This was confirmed by the results of the plate tests in which a definite antagonistic effect was observed. Less pronounced effect was observed with *B. vulgatus* and *Bacillus* sp. No. 390, and little or none with the other species tested.

No interference with the nitrite test for *B. larvae* in the special vegetable extract medium may be expected with non-nitrate-reducing contaminating species. These include not only most types associated with other brood diseases, but also the important *mesentericus* and *vulgatus* groups which may form the most frequent contaminating forms. Nitrate reducing species, e.g., *B. subtilis*, may interfere, though with *B. orpheus* disappearance of nitrite appears to be dependent upon the relative growth of the contaminant and *B. larvae*. It is important that no species tested prevented recognition of *B. larvae* by microscopic tests.

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## COMPARATIVE STUDIES IN POTATO VIRUS DISEASES<sup>1</sup>

BY DONALD F. PUTNAM<sup>2</sup>

### Abstract

This paper is an account of an investigation into the identity of a hitherto undescribed mosaic disease of President potato. Both a mild and a severe form of the disease were observed, but both forms were characterized by a yellow mottling not found in the previously reported potato mosaics.

The mild form of the disease has been shown to be caused by a single virus, while the severe form is due to a combination of this virus and one of the "vein-banding" group. Because of the yellow color associated with the symptoms produced on a number of host plants, the name "yellow mottle" is proposed for the newly described virus.

The "yellow mottle" virus of President mosaic has been compared with the "mottle" and "ringspot" viruses from rugose mosaic, both as to behavior under certain physical and chemical tests and with respect to the symptomatological reaction of a number of solanaceous host plants.

Tabulated results of the differential property studies are given, and descriptions of the symptoms caused by each of the three viruses on eight different host plants are presented.

From these studies it is concluded that the newly described "yellow mottle" virus is distinct from both "mottle" and "ringspot", but it is closely related to the "X-virus" or "latent virus" group.

### Introduction

The potato variety known in England as "President" and in Germany as "Paul Kruger" has for a number of years been grown in Nova Scotia where it was given the name "Never Rot" because of its comparative freedom from late blight rot. It became quite widely grown and valued in those sections where blight injury was prevalent and was eventually entered for inspection under the Potato Certification Service of the Division of Botany, Experimental Farms Branch of the Dominion of Canada Department of Agriculture.

Inspection of the variety brought to light the fact that there existed in much of the stock a rather obscure mosaic disease (Plate I, Fig. 1) which could not definitely be identified with any of the named potato mosaics, although in its full development it bore a resemblance to crinkle and rugose mosaics. The disease appeared to be present in both a mild and a severe form, distinguishable from other types of mosaic by the characteristic yellow cast imparted to the foliage of the infected plants.

This report is based upon work performed in the Department of Botany of the University of Toronto, which was undertaken in order to investigate the nature of the mosaic disease of President and to determine the relationships of the virus or viruses concerned.

<sup>1</sup> Manuscript received December 16, 1936.

Contribution from the Department of Botany of the University of Toronto. Portion of a thesis presented May 1935, in conformity with the requirements for the degree of Doctor of Philosophy.

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### Materials and Methods

Specimens of diseased potato tubers were obtained from various places at the beginning of the investigation, and during its progress a number of additional lots were obtained, largely duplicates from the original sources. The samples usually consisted of from three to six tubers, all of which were grown and examined in the course of the work.

Table I provides a list of the diseased specimens that were included in the comparative studies, together with the source from which they were received.

TABLE I

Source	Variety	Disease
Mr. W. K. McCulloch, Kentville, N.S.	President	Mosaic (mild)
	President	Mosaic (severe)
	Bliss Triumph	Mild mosaic
	Bliss Triumph	Rugose mosaic
	Green Mountain	Mild mosaic
	Green Mountain	Rugose mosaic
Mr. D. J. McLeod, Fredericton, N.B.	Green Mountain	Quanjer's crinkle
	Green Mountain	Mild mosaic
	Green Mountain	Rugose mosaic
	Bliss Triumph	Rugose mosaic
Dr. E. S. Schultz, U.S. Dept. Agr., Washington, D.C.	Green Mountain	Mild mosaic
	Green Mountain	Leaf rolling mosaic
	Green Mountain	Crinkle mosaic
	Green Mountain	Rugose mosaic

A number of other specimen tubers infected with named virus diseases were also received but were not included in the comparative studies beyond the preliminary stages.

Mr. McCulloch also kindly supplied healthy stocks of the four potato varieties: President, Green Mountain, Bliss Triumph, and Irish Daisy. Plants from these specimens and also from some of their progeny which were grown in Simcoe County, Ontario, were used in the inoculation studies.

In addition to the diseased potatoes, two other sources of virus were used in this investigation:

- i. Ordinary tobacco mosaic obtained from the Dominion Tobacco Experiment Station at Harrow, Ontario.
- ii. Veinbanding virus of tobacco from an infected Turkish tobacco plant kindly supplied by Dr. E. M. Johnson of the University of Kentucky.

The majority of the inoculation studies were performed upon solanaceous plants other than potatoes, the seeds of the non-commercial species of which were obtained through the seed exchange service of the Department of Botany.

The work was done in the plant houses of the Department of Botany, the houses having a temperature range of 15°–20° C. during the winter months, but somewhat higher during the spring and summer.

Inoculations, when made directly from plant to plant, were performed by the leaf-rubbing method. Flamed forceps were used to tear off leaflets or bits of leaf tissue, which were then rubbed lightly over the surface of the leaf to be inoculated. When plant extracts or filtered juices were employed, the rubbing was done with sterile forceps holding a small piece of sterile gauze. In some cases where the leaf-rubbing method failed or where infection seemed to be particularly slow, as in the inoculation of potato plants, grafting was employed.

The experiments with aphid transmission were performed in cages covered with cotton or with cotton and cellophane.

### Experimental Procedure

#### PRELIMINARY

The first series of experiments was directed toward a comparison of the symptoms of different potato mosaics on a number of solanaceous hosts in order to determine (i) whether the reactions produced by inoculation with the various specimens of the same name were identical and specific, and (ii) whether the reactions produced by the virus from President mosaic would be specific and sufficient to differentiate it from the viruses of the other diseases.

The preliminary inoculations were carried out, using all the above sources of potato viruses, including apparently healthy potatoes, upon the following list of solanaceous hosts: *Nicotiana tabacum* var. Quesnelle, *Nicotiana alata*, *Nicotiana rustica*, *Petunia hybrida*, *Physalis aequata*, *Nicandra physalodes*, *Datura stramonium* and *Lycopersicum esculentum* var. John Baier; from two to six plants were used in each case. All of these plants, in the seedling stage at least, were found to develop symptoms of virus infection when inoculated from any one of the diseased specimens.

It was further noted on all plants, but especially on *Nicotiana alata*, *N. rustica*, *Datura stramonium* and *Lycopersicum esculentum*, that, when they were inoculated from the diseased President plants, the mottling differed in such a way as to be easily distinguishable from the mottling resulting when these plants were inoculated from other sources of potato viruses.

The chief diagnostic features of this mottling were a more severe yellowing of the intercostal areas of the leaf combined with a green banding effect along the veins. In those cases in which, as was later shown, mixtures of viruses were present, the definite pattern was obscured but the yellow color persisted in scattered patches.

In some cases inoculum from the healthy President plants produced results identical with that from other supposedly healthy potato plants, while in other cases no reaction at all was produced.

Plants inoculated from potatoes having rugose mosaic showed symptoms somewhat similar to those on plants inoculated from severely diseased President potatoes, except for the color difference noted above.

The difference between the mild and the severe disease of President potatoes was seen to be of the same order as the difference between mild and rugose mosaics of Green Mountain and Bliss Triumph; or, for that matter, between apparently healthy Green Mountain and those having rugose mosaic, since no difference in the symptoms upon the test plants could be seen whether they were inoculated from specimens having mild mosaic or from those which were apparently healthy.

It was therefore concluded that the diseased President potatoes contained a virus not found in any of the other diseased specimens and attempts were made to isolate this virus so that comparisons might be made.

#### FILTER ACTION OF *Datura stramonium*

K. M. Smith (10) and Koch (6) both reported that *Datura stramonium* was useful as a filter plant in separating the viruses found in some of the composite diseases of potatoes. It was therefore decided to experiment with the effect of passage upon *Datura stramonium*.

Accordingly, subinoculations were made from all the *Datura stramonium* plants that had been inoculated from the diseased potato plants in the first series of experiments.

The inoculations were made on the following test plants: *Nicotiana tabacum*, *N. alata*, *N. rustica*, *N. glutinosa*, *Hyoscyamus albus*, *Petunia hybrida*, and *Lycopersicum esculentum*; four or five plants of each species being used in each inoculation. Checks were provided by re-inoculations both from the original sources and from the other test plants used in the preliminary experiments.

#### Observations

No difference in the symptoms on the test plants could be distinguished when inoculations were made from *Datura stramonium* plants inoculated either from potatoes affected with mild mosaic or from apparently healthy potatoes. The same was true in the cases where the original inoculation had been from mildly diseased President potato plants.

In rugose mosaic, it was seen that passage through *Datura stramonium* had modified the symptoms on the test plants so that they were identical with those obtained by inoculation from apparently healthy Green Mountain potatoes.

The effect on the virus of the severe disease of President potatoes was similar, in that the symptoms obtained agreed with those resulting after inoculation from mildly diseased President plants.

The symptoms on all the test plants of any one species, inoculated from diseased *Datura stramonium* plants, were similar if the original sources of inoculum were from "mild mosaic", "crinkle mosaic", "Quanjer's crinkle", "leaf rolling mosaic", or "rugose mosaic". Moreover, they agreed very well with those described by Johnson (2) and Koch (6) in the case of the "mottle" virus from rugose mosaic and from apparently healthy potatoes.

The symptoms obtained when the original source of inoculum was diseased President potato plants were distinctly different in all cases from the above, but even after passage through *Datura stramonium* there were variations which suggested that the virus might still be mixed.

Similar experiments were performed with egg plants (*Solanum melongena* var. Black Beauty) in place of *Datura stramonium*, and it was found that the same differentiation of symptoms occurred.

#### FILTER ACTION OF PETUNIA

An attempt was made to duplicate the work of Smith (10) and to isolate a "Y" or "veinbanding" virus by means of the filter action of petunia. In all cases except one it was found that the mosaic complex was transmitted apparently unchanged.

In one case, however, a petunia plant inoculated from a severely diseased President potato plant failed to transmit the virus responsible for the typical yellow mottling on the test plants. Instead, it transmitted a virus which produced symptoms strongly suggestive of the "veinbanding" virus (12). A number of subinoculations were made from this source but the strain was unfortunately lost before complete identification could be made.

#### FURTHER ISOLATIONS FROM DISEASED POTATOES

Some months after the preliminary inoculations reported above, a set of duplicate inoculations was made. For the most part, the symptoms obtained agreed with those which had previously been observed. There were, however, notable exceptions. In three cases the symptom expression on the test plants was not that of the "mottle" virus as described by Johnson (2) but that of "ringspot". The sources of the inoculum were Green Mountain potato plants exhibiting symptoms of leaf rolling mosaic, crinkle mosaic and rugose mosaic.

The "ringspot" virus was found to be capable of passage through *Datura stramonium* plants and was in several cases obtained in pure culture, since subsequent attempts failed to demonstrate the presence of any other virus associated with it.

#### Differential Property Studies

From symptomatological evidence, it was considered that there had been recognized, in the course of the investigation, three distinct viruses which were capable of infecting *Datura stramonium*.

- i. The common "mottle" virus which is carried without symptoms by practically all American potato varieties.
- ii. The "ringspot" virus.
- iii. The "yellow mottle" virus of President mosaic.

The "mottle" virus is apparently often found without contamination in many varieties of potatoes without causing any symptoms; while the "ringspot" virus is apparently seldom found pure in the potato, although it was

accidentally isolated in the course of this investigation. It would seem that the distinctive virus of President mosaic is often found uncontaminated in that variety; but on the other hand, since the common "mottle" virus has also been found alone in President, and a considerable degree of irregularity in the symptoms upon test plants has been observed, it seemed probable that in many cases the ordinary "mottle" virus and the "yellow mottle" virus existed together. In order to establish the purity of the virus isolants, a number of property studies were carried out. The criterion upon which to judge the effect of any treatment is, of course, the symptom expression upon the test plants; and when a point is reached where no further modification takes place in the symptom expression, the virus under consideration is adjudged to be pure.

The tests performed consisted of attempts at insect transmission, investigation of the rate of spread through test plants, and studies on the resistance of the virus *in vitro*.

#### INSECT TRANSMISSION

Preliminary tests were made in order to determine whether any differentiation could be made on the basis of transmissibility by insects. The aphid *Myzus persicae* Sulz. was used in all the tests, healthy experimental stocks being secured by colonizing upon *Datura stramonium* or *Solanum melongena* in cotton cages.

Attempted transfers by means of aphids were made both from infected potatoes and from other solanaceous test plants, but in no case was infection obtained with any of the three viruses. As a check on the technique of these experiments aphid transfers of tobacco mosaic virus and "veinbanding" virus were also attempted and successfully carried out. It was therefore concluded that the "yellow mottle" virus of President mosaic resembled the "mottle" and "ringspot" viruses to the extent that it is not transmissible by the aphid *Myzus persicae* Sulz.

#### RATE OF SPREAD THROUGH THE PLANT

A number of experiments were performed in order to find out how long it takes after inoculation for the virus to reach the upper leaves of a plant. It was hoped that some difference in the rate of spread would be discovered that would help to differentiate the "yellow mottle" virus from the "mottle" and "ringspot" viruses. The plan of experiment in each case was to inoculate a plant on one of the intermediate leaves and then to make daily subinoculations from the tip of the plant until recognizable symptoms appeared.

In one case, the "yellow mottle" virus was obtained from the tips of tomato plants five days after inoculation, whereas definite symptoms of its presence were not visible until the seventh day. In another case, the virus was recovered from the tip of the plant on the eighth day, and the first symptoms were noted on the tenth day. In a similar test with *Nicotiana glutinosa*, the virus was recovered from the tip of the plant on the fifth day, while symptoms appeared on the seventh day.

The "ringspot" virus was obtained from the tips of tomato plants on the third day after inoculation, while the symptoms did not become apparent until the sixth day.

The "mottle" virus required from seven to nine days to reach the tips of inoculated tomato plants, and symptoms became visible in from ten to twelve days. Using *N. glutinosa*, the "mottle" virus was recovered from the tips of the plants in from five to eight days, whereas symptoms appeared in from seven to ten days, although in one case fifteen days elapsed.

The evidence afforded by these experiments would indicate that, in the tomato plant, the "ringspot" virus is able to move most rapidly through the tissues; the virus of President mosaic moves at an intermediate rate, while the ordinary "mottle" or "latent virus" is most sluggish in reaching the growing point.

#### RESISTANCE OF VIRUS EXTRACTS

The tests employed were filtration, dilution, exposure to heat and to various chemicals, and aging *in vitro*.

Filtration was carried out by passing the expressed plant juices through Mandler N candles. The juices were strained through gauze and passed through a pad of non-absorbent cotton to remove the coarser material before filtration. Strained plant extracts were also employed in the other tests.

The exposure to heat was carried out by placing about 2 cc. of a 1:5 dilution of the plant extract in a small thin-walled test tube fitted with a tight rubber stopper and completely immersing it in a water bath held at the required temperature for ten minutes. At the end of the ten minute period the test tubes were immersed in cold water until cool, and the inoculations were made immediately by rubbing with pieces of sterile gauze.

TABLE II  
SUMMARY OF THE DATA ON FILTRATION, DILUTION END POINT, THERMAL DEATH POINT, AND INACTIVATING CONCENTRATION OF CHEMICALS

Test	"Mottle"	"Ringspot"	"Yellow mottle" virus of President mosaic
Filtration: Mandler N	X	X	X
Dilution end point	$\frac{1}{100,000}$	$\frac{1}{10,000}$	$\frac{1}{1,000,000}$
Thermal death point †	68-70° C.	66-68° C.	71-73° C.
Chemicals: ‡‡			
Alcohol	50-75%	50-75%	50-75%
Nitric acid	$\frac{1}{500} - \frac{1}{400}$	$\frac{1}{500} - \frac{1}{200}$	$\frac{1}{500} - \frac{1}{200}$
Formalin	$\frac{1}{50}$	$\frac{1}{50}$	$\frac{1}{100}$
Phenol	2-4%	over 4%	2-4%

† 10 minutes exposure.

‡‡ Concentrations which inactivate after one hour exposure.

The exposure to chemicals was also performed with a 1:5 dilution. The chemicals were all made up to double the required strength and then an equal quantity of the diluted plant juice was transferred by pipette into each test tube. The mixture was allowed to stand for thirty minutes when the first set of plants was inoculated by means of small pieces of sterile gauze, and at the end of an hour a second set of plants was inoculated in the same way.

Table II contains a summary of the data on the filtration dilution, heating and chemical treatments of the "mottle", "ringspot" and "yellow mottle" viruses.

On the whole, the records obtained in the property studies furnish very little basis for separation, with the exception of the thermal death points. Dilution tests are not to be relied upon, for little uniformity of result is ever obtained upon repeating an experiment. All three viruses seem to have equal powers of resistance toward inactivation by various chemicals. With respect to longevity, it was found that President mosaic virus retained its infectivity *in vitro* for more than two months, which compares favorably with the other two viruses. The evidence of these tests all points toward the inclusion of these three viruses as members of the same group.

#### THE BASIS OF SEPARATION

The problem of separation concerns only those residual viruses which are transmitted through *Datura stramonium* since by this passage the "vein-banding" viruses are eliminated. Koch (6) has pointed out that "ringspot" could be separated from "mottle" by virtue of the fact that it was capable of spreading more rapidly in the tissues of the tobacco plant. On the other hand, the "mottle" virus has a higher thermal death point and in this way the "ringspot" virus may be eliminated.

In this investigation similar differences have been found between these two viruses; the thermal death point for the "mottle" virus was 70° C. while that for the "ringspot" virus was 68° C.; the "ringspot" virus was able to reach the tip of a tomato plant in three days, while the "mottle" virus required six or seven days.

The higher thermal death point of the "yellow mottle" virus of President mosaic is enough to separate it from both "mottle" and "ringspot", as the virus which was found infective after heating to 72° C. caused no symptoms upon the test plants attributable to admixture with other strains. It has also been found that sometimes the virus of President mosaic is able to pass more quickly to the tip of an inoculated plant than is the "mottle" virus, but this difference is not constant.

The similarity in the responses of all three viruses to the various physical and chemical tests is evidence that these viruses are closely related. There is no difference to be found in the host range, as no test plant has yet been discovered that will retain any one of them to the exclusion of the others.

The ultimate justification of the differentiation of a complex into separate viruses lies in the fact that once one is obtained pure, by whatever means, the symptom expression on the test plants remains constant through successive subinoculations. All three viruses have remained constant through at least six successive transfers and have shown no tendency to change from one form to the other.

### Symptomatology

The symptoms of the three potato viruses of the "latent" or "X-virus" group encountered in this investigation, upon each of eight different host plants, are described in the following paragraphs.

#### POTATO MOTTLE VIRUS

##### *Tobacco (Nicotiana tabacum)*

No primary symptoms were observed on the inoculated leaves of seedling plants, but vein clearing followed by a very mild mottling appeared on the young leaves in from two to three weeks. As the plants grew older the intensity of the mottling decreased until at flowering time no symptoms could be seen. No appreciable stunting of the plants took place.

##### *Flowering Nicotine (N. alata)*

Yellowish lesions appeared, in some cases, on the inoculated leaves in from ten days to two weeks, followed closely by vein clearing and a definite mottling of light and dark green, the darker color usually existing in patches along the veins. Neither primary nor secondary necrosis was seen. It was much more difficult to infect plants after they had passed the seedling stage.

##### *Nicotiana rustica*

Usually no primary symptoms were observed, although occasionally small lesions appeared at the point of inoculation. Clearing of the veins of the youngest leaves was observed about a week after inoculation, followed by a mild mosaic mottling of light and dark green areas without any sign of necrosis or distortion (Plate I, Fig. 2). The mottling faded out in the mature plants.

##### *Nicotiana glutinosa*

Primary symptoms were not observed. Secondary symptoms appeared in about ten days, the mottling being very mild and often almost unnoticeable (Plate I, Fig. 3). If the plants were inoculated as very young seedlings a slight stunting was noticed, but on older plants the effect was negligible.

##### *Hyoscyamus albus*

As a rule there were no primary lesions. Early secondary symptoms consisted of an indefinite clearing of the veins, while later symptoms consisted of a mild mottling sometimes accompanied by interveinal necrosis (Plate I, Fig. 4).

##### *Petunia hybrida*

No primary symptoms were observed, but mottling appeared in from two to three weeks. No stunting or necrosis was noted, the disease being of a very mild type.

*Tomato (*Lycopersicum esculentum*)*

A very mild mottling made its appearance in from one to three weeks, depending upon external conditions. As a rule the whole plant had a slightly yellowish or chlorotic appearance. The mottling usually consisted of dark green bands along the veins with light green intercostal areas. In older plants the symptoms tended to disappear, although a slight stunting sometimes occurred.

*Jimson Weed (*Datura stramonium*)*

No primary symptoms were seen but a faint mottling appeared in from five to ten days. This was usually of a mild veinbanding type which in some cases tended to become more intense (Plate I, Fig. 5).

## POTATO RINGSPOT VIRUS

*Tobacco (*Nicotiana tabacum*)*

Small white-ringed necrotic lesions appeared on the inoculated leaves in from three to five days. The necrosis spread rather rapidly, especially at high temperatures, and often destroyed most of the tissue of the leaf. Secondary symptoms appeared on the upper leaves in from seven to ten days after inoculation and consisted of very numerous ringspots, each of which was about 1 mm. in diameter (Plate I, Fig. 6). These central rings became surrounded by larger rings and portions of larger rings, until very little green tissue was left. Later leaves were less severely affected, and in old plants the symptoms were reduced to a rather indefinite mottling interspersed with faint wavy lines. Tobacco seedlings were inoculated in April and kept in the greenhouses all summer, and in the fall no symptoms of any sort were exhibited but the virus was easily recoverable from the leaf tissues by inoculation upon other test plants.

*Flowering Nicotine (*N. alata*)*

Primary, white, necrotic ringspots appeared in five or six days. On young seedlings, the inoculated leaves soon became completely necrotic and dropped off. Secondary symptoms appeared in from one to two weeks after inoculation, usually beginning with clearing and necrosis of the younger leaves. This was followed by varying degrees of necrosis of the ring-and-line type (Plate I, Fig. 7). Later leaves usually displayed a milder type of necrosis and the upper part of the plant exhibited only a mottling which became less intense as the plant matured. Young plants were sometimes killed by systemic necrosis whereas older plants exhibited only a mild mottling in addition to the primary lesions on the inoculated leaf.

*Nicotiana rustica*

This plant was found to display the symptoms of ringspot to their best advantage. Primary symptoms sometimes appeared in less than three days after inoculation. At first they consisted of very small white rings on the inoculated surface and were not visible through the leaf. Within a day they deepened so as to be seen from the other side of the leaf and also became

surrounded by a second white ring (Plate I, Fig. 8). This process was repeated until the spots coalesced to form an irregular pattern of concentric lines. The inoculated leaf, especially in young seedlings, often became completely necrotic and was dropped. In older plants, only the inoculated portions of the leaf became necrotic. Secondary symptoms were noted on the young leaves, at the growing tip, in from one to two weeks after inoculation. Here the necrosis was usually in the form of an irregular ring-and-line pattern rather than small discrete rings as in the case of tobacco. Later, wide necrotic margins were noted along the principal veins of some of the leaves behind the growing point. The symptoms reached their full development on the intermediate leaves produced just subsequently to the systemic spread of the virus. Mottling, as generally understood, was not present. As a rule, bands of dark green tissue persisted along the chief lateral veins, but the intercostal areas were occupied by a zonate pattern of white necrotic lines, visible from both sides of the leaf (Plate I, Fig. 9). The upper leaves of these plants were usually symptomless or, at most, bore only a few small ringspots.

The younger the plant when it is inoculated, the more general is the necrotic effect of the virus. As the plant grows older it seems to develop some sort of resistance to the spread of the virus, for plants inoculated after the flower buds were formed produced only primary ringspots and partial necrosis of the inoculated leaf. That the virus did not become systemic was shown by the fact that it could not be recovered except from the inoculated leaf. In such cases, no immunity is conferred upon the rest of the plant, for the upper leaves of some of these plants were inoculated at a later date and primary lesions were produced.

#### *Nicotiana glutinosa*

Both primary and secondary symptoms on this plant resemble those of *N. rustica* except that there is a tendency for the secondary symptoms to be more irregularly necrotic, and to exhibit less of the ring-and-line pattern (Plate II, Fig. 10). In some cases it was found that the necrosis became systemic and killed the plants. The survivors exhibited a mottling, which gradually became less intense, and they were always very much stunted as compared with uninoculated control plants.

#### *Hyoscyamus albus*

Primary necrotic lesions appeared on the inoculated leaves in three or four days. In about two weeks a rather intense secondary necrosis was noted at the growing point, while at the same time discrete lesions appeared upon the intermediate leaves (Plate II, Fig. 11). The necrosis of the growing point gradually became less intense, and eventually the only symptom exhibited by the young leaves consisted of a mild mottling.

#### *Petunia hybrida*

No definite primary lesions were noted, but there were slightly discolored regions on the inoculated leaf. Secondary symptoms appeared very slowly as compared with those on other plants, but mottling and secondary necrosis

were observed about four weeks after inoculation. The plants sometimes recovered from the necrotic stage and produced mottled leaves.

The symptoms on petunia do not suggest the ringspots of other plants, but subinoculations produced typical symptoms on tobacco and *N. rustica*.

#### *Tomato (*Lycopersicum esculentum*)*

Primary local necrotic lesions were noted on the inoculated leaves in from three to five days, while vein clearing and fine necrotic spots appeared on the upper leaves within the week (Plate II, Fig. 12). These leaves always became necrotic and fell off; sometimes the whole top of the plant became necrotic and, in extreme cases, the plants died.

Usually, however, recovery takes place, and in about four weeks new leaves appear, which are only mildly necrotic but have a rather irregular mottling. (Plate II, Fig. 13). The symptoms gradually become less intense as the plant matures; the necrosis disappears, followed by the mottling, so that the upper parts of the plant may have the appearance of complete recovery. Subinoculations from the recovered parts, however, were found to transmit the virus in as virulent a form as ever.

#### *Jimson Weed (*Datura stramonium*)*

Small primary ringspots appeared on the inoculated leaves in six days. The inoculated leaves became completely necrotic and were dropped within a short time. The secondary symptoms depend a great deal upon the age of the plant. In seedlings, secondary necrosis may develop within three weeks and kill the plant. Older plants were almost completely defoliated, but put out new leaves exhibiting a rather intense yellow mottling with marked distortion and ruffling.

### POTATO YELLOW MOTTLE VIRUS

#### *Tobacco (*Nicotiana tabacum*)*

The symptoms produced by this virus on the tobacco plant are not much more severe than those caused by the "mottle" virus. The mottling is somewhat more fine grained and may even tend toward half-rings in some cases (Plate II, Fig. 14). The small intercostal areas have a definite yellow color. As the plant matures the symptoms become less conspicuous, and old plants may be entirely symptomless. There is very little stunting.

#### *Flowering Nicotine (*Nicotiana alata*)*

The primary symptoms usually consisted of yellowish lesions on the inoculated leaves, often followed by a diffuse necrosis. In about a week after inoculation the upper leaves exhibited a yellowish clearing of the veins, followed by mottling. In some cases the mottled leaves bore small necrotic spots. An extreme and very typical mottling was observed after about three weeks (Plate II, Fig. 15). It consisted of dark green bands along the veins and very light green or greenish-yellow intercostal areas. The mottling has an angular appearance not seen in the case of ordinary tobacco mosaic or other potato mosaic viruses. The mottling becomes much less conspicuous in older plants but was not observed to disappear entirely.

*Nicotiana rustica*

No primary symptoms appear on the inoculated leaves, but in a week or ten days necrotic spots appear on the young leaves at the growing tip. This is followed by a severe and definitely marked mottling with a few necrotic spots on the mottled leaves (Plate II, Fig. 16). As the plants mature the mottling becomes less severe and the young leaves do not exhibit necrosis.

*Nicotiana glutinosa*

Except for occasional slightly yellowed areas, primary symptoms do not appear on the inoculated leaves. Extreme clearing of the veins occurs in the young leaves in from eight to ten days, followed by a severe yellowish mottling of the same type as that observed upon *N. rustica* (Plate II, Fig. 17). Necrotic spots are frequently present, and the mottled leaves become senescent rather rapidly. The plants are usually very much stunted and often become almost completely defoliated.

*Hyoscyamus albus*

As a rule no primary symptoms were observed on the inoculated leaves. Secondary symptoms consisted of a mottling on the younger leaves which was usually somewhat more severe than that caused by the "mottle" virus (Plate II, Fig. 18).

*Petunia hybrida*

No primary symptoms were observed and the secondary symptoms were usually very slow in appearing. No necrosis occurred but the plants displayed a mild yellow mottling some weeks after inoculation (Plate II, Fig. 19).

*Tomato (*Lycopersicum esculentum*)*

Very specific effects were obtained with this virus upon tomato so that it was possible to distinguish it without difficulty from all the other viruses encountered.

In from five to seven days a mild yellow vein clearing appears on both the inoculated leaf and the young leaves at the top of the plant. In some cases there is necrosis of the young leaves, although usually the leaves showing clearing of the veins afterward expand normally and show the typical yellowish mottling. This mottling is much more extreme than the mottling caused by the "mottle" virus. The mottled leaves exhibit dark green bands of tissue along the veins while the intercostal areas are quite yellow, or in some cases bleached completely (Plate III, Fig. 20). If the plants are inoculated while very young, there is some tendency toward rugosity and rolling of the leaves, but in older plants there is no distortion. Badly infected plants are stunted and show little tendency toward recovery, although the symptoms become less distinct in mature plants.

*Jimson Weed (*Datura stramonium*)*

The first sign of disease is the clearing of the veins of the younger leaves, followed by a faint mottling. The mottling is sometimes produced by bands of dark green, but the most pronounced effect is usually a dark green spotting.

Later the mottling becomes more irregular, the chlorotic areas become yellow, and there is considerable distortion accompanied by small necrotic areas. The plants are stunted and the whole effect of the disease is more severe than after inoculation with either of the other two viruses (Plate III, Fig. 21).

### Description of the Viruses

The communications of Johnson (3), Valleau and Johnson (12) and Koch (6) contain descriptions of the "mottle", "ringspot" and "veinbanding" viruses. In a recent publication, Johnson and Hoggan (4) have discussed the matter of virus identification and have indicated what they consider should constitute an adequate description of a filtrable virus. For the purposes of comparison their form will be followed in the summary descriptions of the three viruses isolated in the course of this work.

*Potato mottle virus.* Type: Johnson, J., Wisconsin Agr. Expt. Sta. Res. Bull. 63, 1925. Not transmissible by means of the aphid *Myzus persicae* Sulz. Readily transmissible by means of needle, tissue rubbing or graft inoculation. Filtrable readily through Mandler N and Berkfeld W candles. Longevity *in vitro*, several months. Thermal death point 70° C., for ten minutes. Host range: Potato (*Solanum tuberosum* L.), Tobacco, (*Nicotiana tabacum* L.), Nicotine (*N. alata* L.), Jimson weed (*Datura stramonium* L.), and a great many other members of the Solanaceae. Symptomatology: Mild mottling of green and light green shades on the foliage of most solanaceous plants in the younger stages. In older plants the mottling is often obscured. In most American potato varieties the virus is carried without symptom expression. Distribution coexistent with potato culture.

*Potato ringspot virus.* Type: Johnson, J., Wisconsin Agr. Expt. Sta. Res. Bull. 63, 1925. Not transmissible by means of the aphid *Myzus persicae* Sulz., but readily transmissible mechanically by means of plant juices or by grafting. Filtrable readily through Mandler N and Berkfeld W candles. Longevity *in vitro*, several months. Thermal death point, 68° C., for ten minutes. Host range: Potato (*Solanum tuberosum* L.), Tobacco (*Nicotiana tabacum* L.), Nicotine (*N. alata* L.), *N. rustica* L., *Datura stramonium* L., and many other solanaceous plants. Symptomatology: Distinctive, small ring-shaped necrotic spots at the point of inoculation on tobacco, *N. alata*, *N. rustica*, and *Datura stramonium* followed in a few days by the development of ringspots on the upper leaves of the plants. Best characterized by the development of a remarkable ring-and-line pattern on the leaves of *N. rustica*. In most American potato varieties this virus is carried without symptoms. Distribution coexistent with potato culture although not so frequently encountered as the potato mottle virus.

*Potato yellow mottle virus* (President mosaic virus). Type: this paper. Not transmissible by the aphid *Myzus persicae* Sulz. but readily transmissible mechanically by plant extract and by grafting. Filtrable readily through Mandler N and Berkfeld W candles, and with difficulty through Seitz E. K.

size 3 discs. Longevity *in vitro*: two months or more. Thermal death point: 73° C. for ten minutes. Host range: potato (*Solanum tuberosum* L.), tobacco (*Nicotiana tabacum* L.), Nicotine (*N. alata* L.), tomato (*Lycopersicum esculentum* L.), Jimson weed (*Datura stramonium* L.), and many other solanaceous plants. Symptomatology: slightly yellowish interveinal mottling on President potato, and an indefinite mottling on tobacco, but best characterized by a strikingly regular mottling on tomato in which there are bright yellow interveinal areas and very dark green bands of tissue along the veins (Plate III, Fig. 20). Distribution: reported only from the variety President grown in Nova Scotia.

### Re-inoculation and Re-isolation

In order to complete the investigation it is necessary to reproduce the original disease by re-inoculation of healthy specimens of the original host with the isolated viruses, and then to re-isolate the same viruses. This is difficult, for obvious reasons, and doubly so when the original host is the potato which may contain so many "latent" or hidden viruses, each of which may be able to influence the reaction.

#### RE-INOCULATIONS ON POTATO PLANTS

The plants used in the tests were grown from tubers obtained from Nova Scotia as healthy, or from their progeny grown in tuber units in Ontario. Each tuber unit used was tested for the presence of "latent" viruses. In all cases, Green Mountain, Bliss Triumph and Irish Daisy contained the "mottle" virus while the President plants apparently contained no sap-transmissible viruses. The following is a record of the series of re-inoculations on potato.

1. Rugose mosaic was inoculated into apparently healthy Green Mountain plants directly from Green Mountain potatoes, and after passage through tomato, tobacco *Nicotiana alata*, petunia, and *Datura stramonium*. At the same time, direct inoculations were made from an apparently healthy Bliss Triumph potato and from mottled tobacco and tomato plants which had been inoculated from it.

The plants inoculated with rugose mosaic, both directly and after passage through tomato, tobacco, *N. alata* and petunia, all developed severe current-season symptoms of rugose mosaic and died within a month. The potato plants inoculated from apparently healthy Bliss Triumph, or test plants inoculated from it, remained apparently healthy, as also did those inoculated with rugose mosaic after passage through *Datura stramonium*.

2. A number of attempts have been made to inoculate Green Mountain and Bliss Triumph potatoes with the mosaic from President potatoes, both directly and after passage through various test plants, but in no case have any symptoms been observed. In nearly all cases attempts were made to re-isolate the virus from the inoculated plants, but to date there is no evidence that Green Mountain or Bliss Triumph potatoes either become diseased or act as symptomless carriers toward the virus from President potatoes.

3. The "ringspot" virus from diseased tomato plants was inoculated into four Green Mountain, four Bliss Triumph, and four President plants. No apparent symptoms were obtained upon the inoculated plants. Attempts at re-isolation of the virus were made by taking a leaf from each of the four plants in each series, macerating them together, and inoculating two *Nicotiana rustica* plants from each series. Mild "ringspot" symptoms were obtained in all cases, and in addition, typical "mottle" symptoms were obtained from the Green Mountain and Bliss Triumph varieties.

4. Twelve President potato plants were inoculated with the virus of President mosaic after passage through *Datura stramonium*. All of them became diseased and exhibited a mottling identical with that of the original specimens. The tubers from one of these plants were grown again in the greenhouse and exhibited typical symptoms.

5. Eight President potato plants were inoculated with the virus obtained by passage of the President mosaic through petunia. These plants all showed a mild mottling about a month after inoculation, rapidly followed by a yellowing or premature senescence of the whole plant, which soon succumbed.

6. Four President potato plants were inoculated with a combination of the virus of President mosaic after passage through *Datura stramonium*, and the virus obtained by passage through petunia. The typical President mottling appeared first in the tip leaves of all the plants. Following that, three of the plants began to turn yellow and succumbed in the same manner as those inoculated with the petunia virus alone.

7. "Veinbanding" virus from the tobacco plant supplied by Dr. E. M. Johnson was inoculated upon two plants each of the President, Bliss Triumph, Irish Daisy, and Green Mountain varieties. Infection was obtained on all the inoculated potatoes with the exception of one President plant. The symptoms upon President consisted of necrosis and abscission of the inoculated leaf followed in about three weeks by the appearance of pale patches on the upper leaves. In the case of Bliss Triumph variety a mottling appeared in the upper leaves of both plants, followed by a yellowing and necrosis of the leaves much like that observed when President plants were inoculated with the virus obtained by passage through petunia. The symptoms resembled those reported by previous investigators. The symptoms on the Green Mountain and Irish Daisy varieties were similar.

#### RE-ISOLATION FROM ARTIFICIALLY INOCULATED POTATO PLANTS

Re-isolations on test plants were attempted in all the foregoing cases in which the viruses were re-inoculated on potato. In only one case, however, was it possible to demonstrate, by the re-isolation of the virus in a pure state that a typical mosaic disease had been caused by the inoculation of a potato plant with a single virus. In all other cases, either viruses were recovered from apparently symptomless plants, or else those plants which exhibited symptoms were shown to contain virus mixtures.

The President mosaic virus used in the re-inoculation on potato was obtained from a tomato plant which exhibited very characteristic and unmistakable symptoms. After symptoms had appeared on the potato plants, a series of re-isolations were made on tomato plants which exhibited the same typical mottling without any modification.

Thus it is possible to present a complete chain of evidence, including isolation, re-infection and re-isolation, to show that a single virus entity is able, unassisted, to cause a specific mosaic disease on the President variety of potatoes. This can be done, only because of the fact that there exist stocks of the President variety that are free from the "latent" viruses.

### The Recombination of Separate Viruses into Artificial Associations

#### COMBINATIONS CONTAINING THE "VEINBANDING" VIRUS

Since the rugose mosaic complex was found to produce a standard necrotic disease on *Nicotiana alata*, it was deemed advisable to make comparisons on this plant by inoculation with combinations comprising the "veinbanding" virus and each of the three residual viruses. The reactions of the test plants are given in Table III. One set of plants was inoculated directly with rugose mosaic from a potato plant as a control. The two viruses in each combination were rubbed on opposite halves of the same leaf on each plant.

TABLE III  
RESULTS OF INOCULATION OF *Nicotiana alata* WITH VIRUS COMBINATIONS

Inoculum	The reaction on four <i>N. alata</i> seedlings
Rugose mosaic (control)	4 plants, vein clearing and necrosis
"Veinbanding" and "mottle"	4 plants, vein clearing and necrosis
"Veinbanding" and "ringspot"	3 plants, vein clearing and necrosis 1 plant, ringspot only
"Veinbanding" and "yellow mottle" virus from President mosaic	4 plants, vein clearing and necrosis

The symptoms of the combination diseases appeared much sooner than did the vein clearing upon *N. alata* plants inoculated with the "veinbanding" virus alone. The plants inoculated with "ringspot" in combination with the "veinbanding" virus exhibited symptoms a day earlier than the other combinations, but all showed symptoms within a week after inoculation.

The clinical picture of the early stages of the disease was much the same on all the plants, and resembled closely that observed on *N. alata* seedlings when inoculated from severely diseased President potatoes. Complete necrosis of the central leaves together with a partial necrosis along the veins of older leaves took place in all cases (Plate III Figs. 22-25). In later stages, those plants which were inoculated with combinations containing either the "ringspot" virus or the "yellow mottle" virus from President mosaic appeared to be more severely diseased.

The success of these experiments in synthetically reproducing the original disease picture in each case is conclusive evidence that the severe disease of President was caused by a virus complex. It is also further evidence of the relationship of the residual virus of President mosaic to the group of "latent" or "X-viruses".

#### TOMATO STREAK

Interest in the "streak" disease of tomatoes was aroused quite early in this investigation by the discovery of a typically diseased tomato plant in a group which had been inoculated from diseased President potato plants. The symptoms exhibited by this plant agreed quite well with those described by Vanterpool (13), and it was thought that the disease had been caused by accidental contamination from an adjoining house containing plants infected with tobacco mosaic. Accordingly an attempt was made to analyze the complex by inoculation on *Nicotiana glutinosa*, which successfully eliminated the tobacco mosaic virus. At the time, although it was not certain that pure cultures of the various potato mosaic viruses had been isolated, a preliminary inoculation series was performed in which twelve different isolations from diseased potato plants were separately combined with the virus of tobacco mosaic, and inoculated on tomato plants which were about a foot high. All the plants became diseased and showed "streak" symptoms varying from a slight degree of wilting to systemic necrosis and death of the plant. A number of experiments were performed in the hope of determining the cause of this great variation, but without success.

At a later date, after the identity of all three separate residual viruses from potatoes had been established, further experiments were undertaken in order to study the effect of combining each of these residual viruses with the tobacco mosaic virus upon the tomato plant. It was found that all three combinations produced a typically necrotic disease on tomato. The disease produced by the combination of tobacco mosaic virus and the ordinary "mottle" virus, however, was slightly less virulent than the other two. This, of course, might be expected, as both the "ringspot" and the "yellow mottle" viruses are able at times to cause necrotic symptoms on the leaves of tomato.

The symptoms of tomato "streak" have been well illustrated by other investigators, but as this record is an addition to the number of viruses that can be combined with the virus of tobacco mosaic to cause "streak", photographs of symptoms are appended (Plate III, Figs. 26, 27). Those plants which recovered from the necrotic phase of the disease exhibit a strikingly yellow mottling and rugosity of the foliage; occasionally also, leaflets appear upon which are exhibited symptoms typical of one or other of the component viruses.

It was noted also, when this mixture of viruses was inoculated on tomato plants already exhibiting the "mottle" disease, that the symptoms were not so severe, and the recovery was more rapid than in the case where the inoculation was made on healthy plants.

The fact that all three residual viruses are able, in combination with the virus of tobacco mosaic, to cause typical "streak" of tomatoes is further evidence that they are related. The evidence that "streak" is less virulent if the tomato plants are already infected with the "mottle" virus, is in agreement with the statement of Ainsworth (1), and might be said to indicate a relationship between the potato virus fractions of the "streak" virus.

#### COMBINATIONS OF RESIDUAL VIRUSES

In the preliminary experiments there was evidence to indicate the existence in potato plants of natural mixtures containing the "mottle" and "ringspot" viruses, as well as the "mottle" and "yellow mottle" viruses. It was felt that an attempt should be made to reconstitute these observed mixtures from isolated viruses; and, although such a mixture had not been detected, a similar attempt was made to produce a combination of the "ringspot" and "yellow mottle" viruses.

Tomato plants about a foot in height were used for the experiment and the inoculations were made simultaneously with each pair of viruses on opposite halves of the terminal leaflet of an intermediate leaf.

In order that a complete series of reactions might be observed, the following combinations were employed; "mottle" and "ringspot", "mottle" and the "yellow mottle" virus from President mosaic, and "ringspot" and the "yellow mottle" virus. As soon as the first symptom became apparent subinoculations were made from the inoculated leaflets and from the tip of the plant in each case. The symptoms which developed upon the upper parts of the plants, and upon the plants subinoculated from their tips, showed that both viruses became systemic in each case. On the other hand, the viruses remained unmixed in the inoculated parts.

The symptoms, in the "mottle" and "ringspot" combination, consisted of a mild mottling, indistinguishable from that observed on tomato plants infected with the "mottle" virus, or the later stage of the "ringspot" disease. Subinoculations upon *Nicotiana rustica* proved that the "ringspot" virus was present with the "mottle" virus.

In the two other combinations an irregular yellow and green mottling was caused on the upper parts of the plant and on the plants subinoculated from their tips (Plate III, Fig. 28). The mottling was slightly more intense on the plant inoculated with the combination of "ringspot" and "yellow mottle" viruses. The "ringspot" virus was obtained in a pure state from this combination by means of needle inoculation from the green portions of the leaf; while rubbing with bits of crushed tissue transmitted a combination disease. Unfortunately, it was not possible to make a study of the localization of the viruses in other cases.

In another attempt to synthesize a combination disease, two tomato plants were inoculated with the "mottle" virus; and after systemic symptoms had appeared, they were again inoculated with the virus from President. The later symptom expression of both plants was changed and an irregular yellow mottling appeared in place of the mild green mottling caused by the first

inoculation. Subinoculations from these plants reproduced the irregular symptoms upon other tomato plants, but it was noted that the mottling became somewhat more fine-grained and approached quite closely in appearance to the symptoms produced by direct inoculation from mildly diseased President potato plants.

### Discussion

From the above experiments and observations it is evident that the diseased President potato plants were infected by a virus differing from any that could be found in the standard named types of potato mosaics that were available for comparison. From its behavior in regard to insect transmission and passage through *Datura stramonium* it is evidently related to the "mottle" and "ringspot" viruses of Johnson (2) and Koch (6), or the "latent" and "virulent latent" viruses of Jones, Anderson and Burnett (5). In its ability to produce a mosaic disease of potato alone and unaided, it shows affinities with the "simple mosaic" virus of Murphy and M'Kay (8). Its behavior under exposure to certain physical and chemical tests is an additional reason for regarding it as a member of the group which may be termed after Smith (10) "X viruses".

This investigation has produced no evidence to show that any of the viruses of this group may change form by passage through different host plants. On some hosts the symptoms are indistinguishable, but upon transfer to a suitable host the distinguishing symptoms always reappear. Certain plants have been found to exhibit distinctive symptoms which are specific for the identification of certain viruses; the symptoms of "ringspot" appear most characteristically on *Nicotiana rustica* while those of the "yellow mottle" virus of President mosaic are most fully developed on the tomato. It may therefore be necessary to work with a fairly extended list of host plants in order to differentiate completely all the members of a virus group. The difference in the severity of the symptoms between the mild and severe types of the mosaic disease of President is of the same order as that shown in the case of the mild and rugose mosaics of Green Mountain potatoes. Koch (6) has shown that rugose mosaic is a combination disease caused by infection with either the "mottle" or the "ringspot" virus and a "veinbanding" virus. Schultz, Bonde and Raleigh (9) report that mild mosaic is caused by an aphis-borne virus and the "latent mosaic virus". The difference between the two diseases is therefore due to the difference between the two aphis-borne viruses. In the severe disease of President potatoes it was found that a virus of the "veinbanding" type was present, but the mild disease is caused by a single virus. The condition observed in the President variety in Nova Scotia is parallel to that described overseas where Murphy and M'Kay (8) have shown that the "crinkle" of Murphy (7) is caused by a compound infection with the virus of "simple mosaic" and "virus A".

Experimentally at least, tomato streak may be caused by a combination of the "yellow mottle" virus with tobacco virus 1; this is further evidence of its relationship with the "latent" viruses. The behavior of the "yellow mottle"

PLATE I

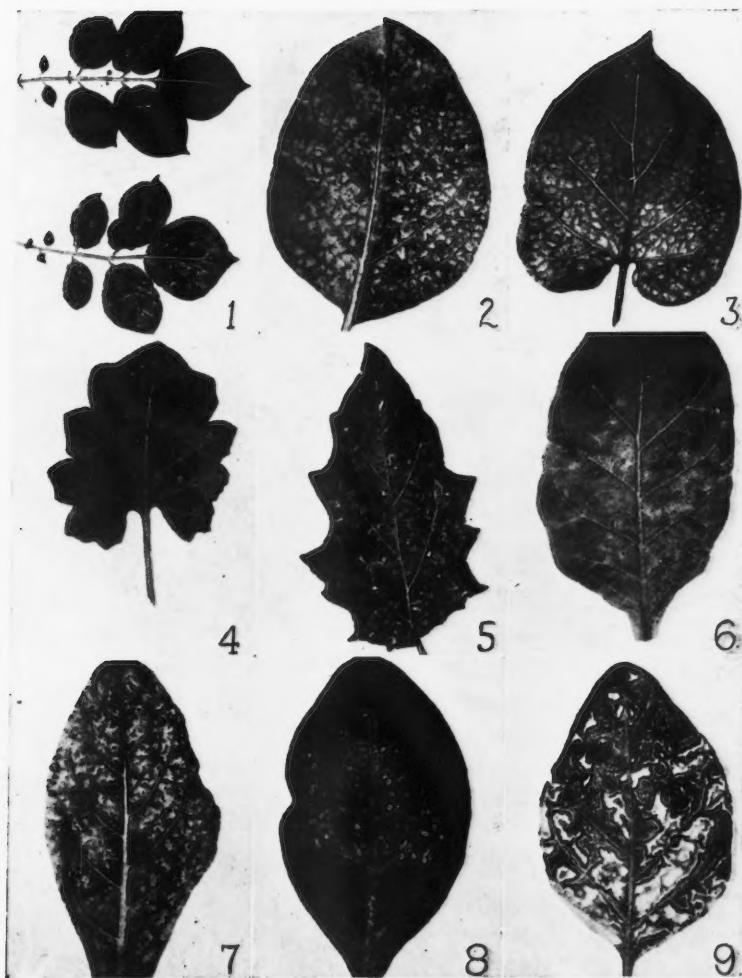


FIG. 1. Healthy and diseased leaves of President potato. FIG. 2. Leaf of *Nicotiana rustica* infected with the "mottle" virus. FIG. 3. Leaf of *N. glutinosa* infected with the "mottle" virus. FIG. 4. Leaf of *Hyoscyamus albus* infected with the "mottle" virus. FIG. 5. Leaf of *Datura stramonium* infected with the "mottle" virus. FIG. 6. Tobacco leaf about ten days after inoculation with the "ringspot" virus. FIG. 7. Typical symptoms of "ringspot" upon an intermediate leaf of *N. alata*. FIG. 8. Leaf of *N. rustica* three days after inoculation with the "ringspot" virus. FIG. 9. Fully developed symptoms of "ringspot" upon an intermediate leaf of *N. rustica*.

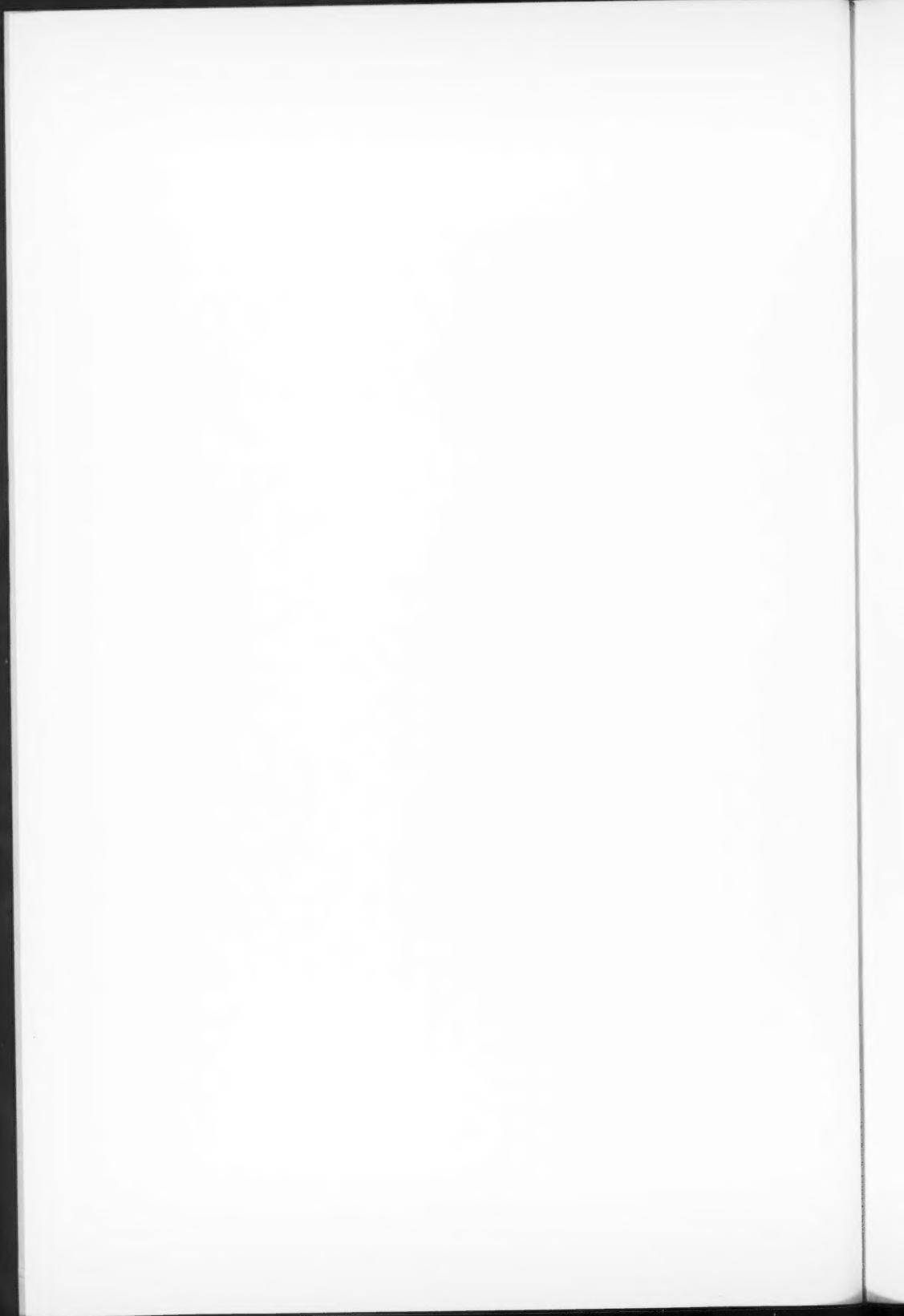


PLATE II

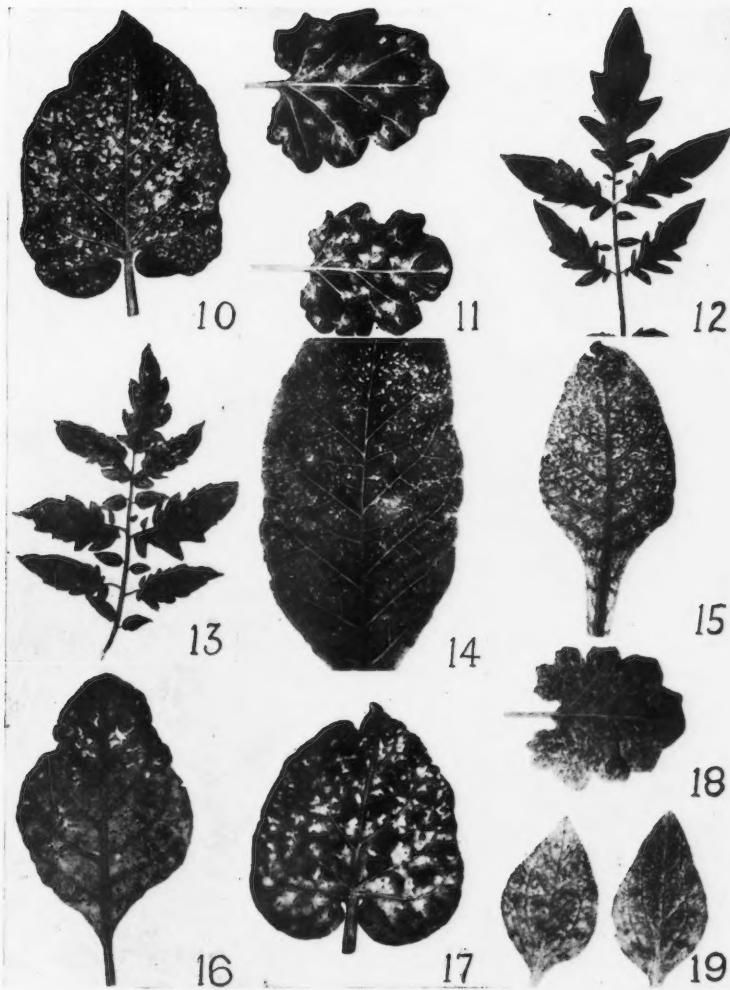


FIG. 10. Leaf of *N. glutinosa* showing symptoms of "ringspot". FIG. 11. Leaves of *Hyoscyamus albus* showing symptoms of ringspot. FIG. 12. "Ringspot" symptoms on a young tomato leaf. FIG. 13. Tomato leaf showing symptoms of a later stage of the "ringspot" disease. FIG. 14. Leaf from tobacco plant infected with the virus from President potato. FIG. 15. Leaf of *Nicotiana alata* about three weeks after inoculation with the virus from President potato. FIG. 16. Leaf of *N. rustica* infected with the virus from the President potato. FIG. 17. Leaf of *N. glutinosa* infected with the virus from President potato. FIG. 18. Leaf of *Hyoscyamus albus* infected with the virus from President potato. FIG. 19. Petunia leaves showing symptoms of infection with the virus from President potato.

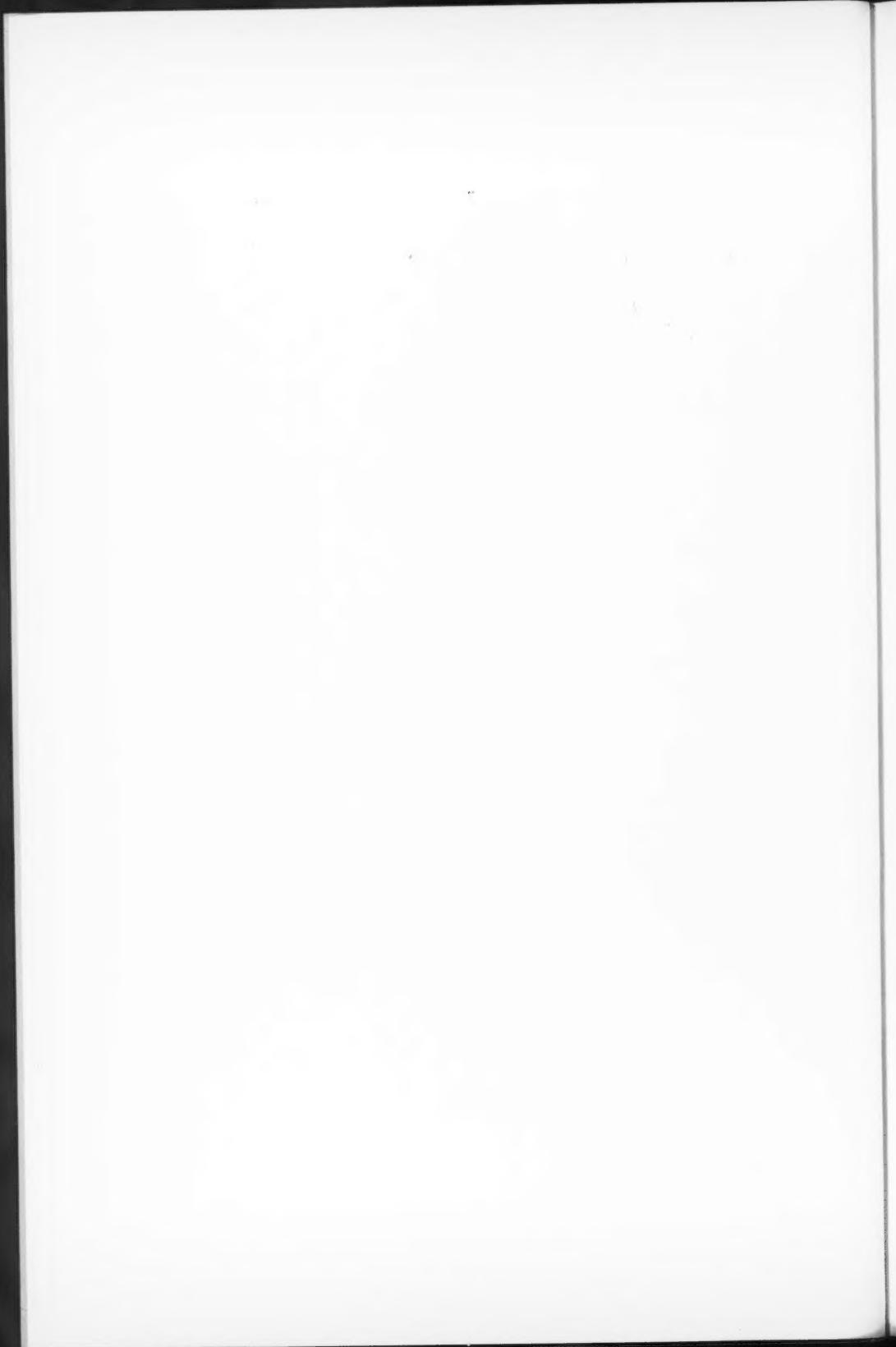


PLATE III

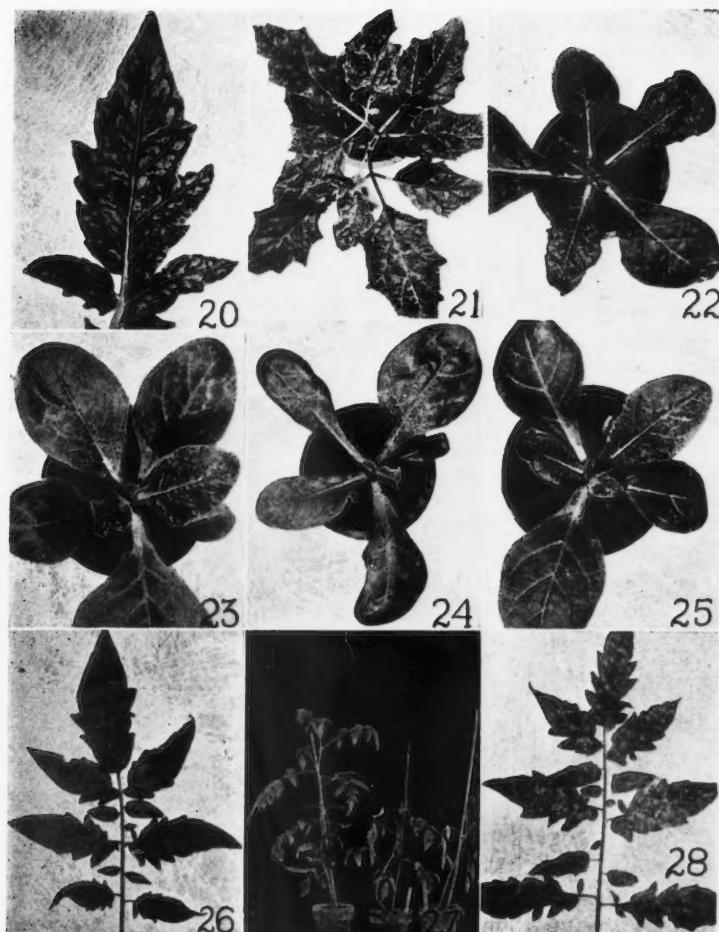
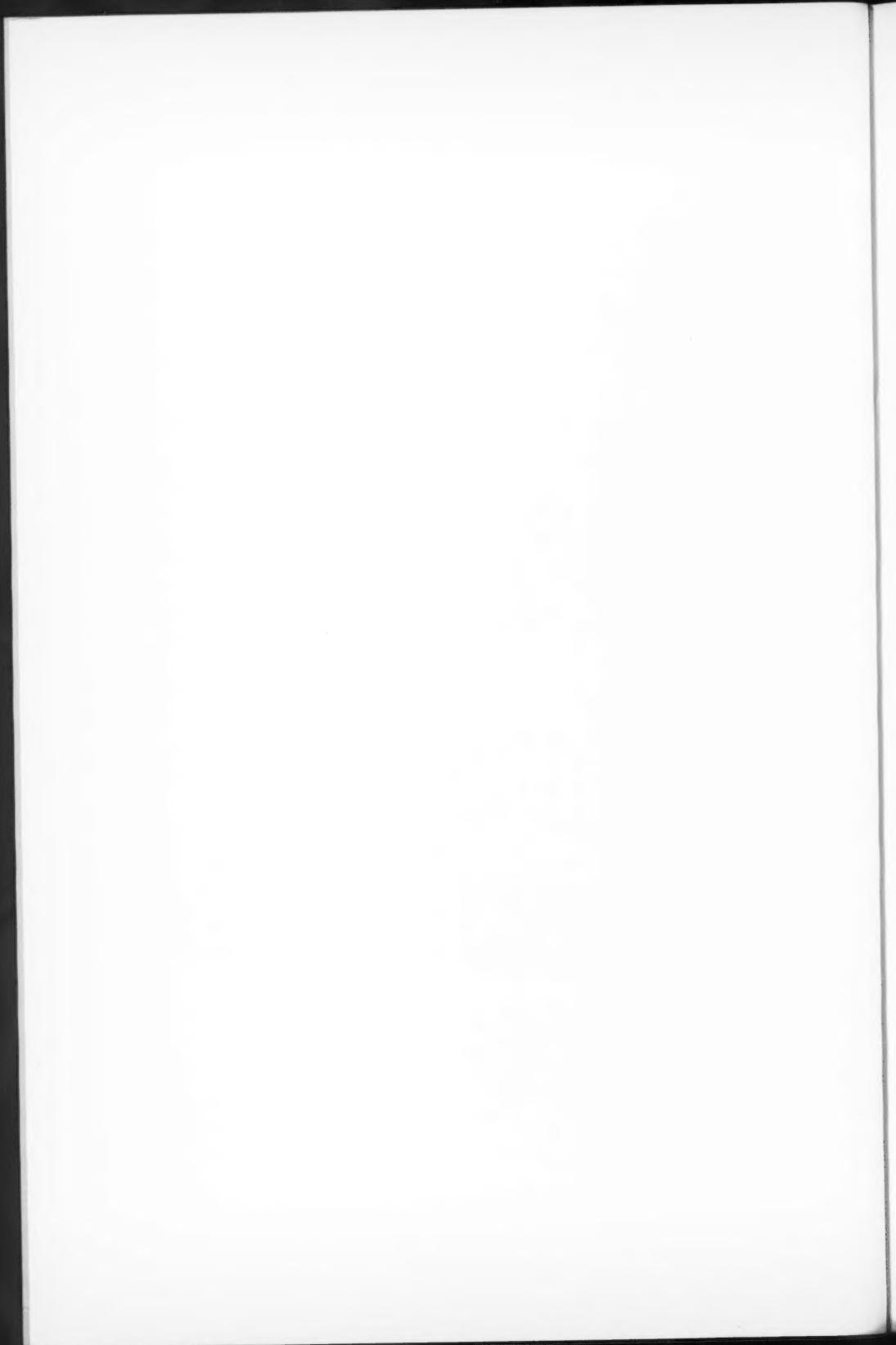


FIG. 20. Leaflet from tomato plant showing symptoms of infection with the virus from President potato. FIG. 21. *Datura stramonium* plant infected with the virus from President potato. FIG. 22. *Nicotiana alata* plant ten days after inoculation with rugose mosaic. FIG. 23. *N. alata* plant ten days after inoculation with the "veinbanding" and "mottle" viruses. FIG. 24. *N. alata* plant ten days after inoculation with the "veinbanding" and "ringspot" viruses. FIG. 25. *N. alata* plant ten days after inoculation with the "veinbanding" and President mosaic viruses. FIG. 26. Tomato leaf showing early symptoms of "streak". FIG. 27. Tomato plants showing "streak" and healthy control. FIG. 28. Leaf of tomato plant inoculated simultaneously with the viruses of "ringspot" and President mosaic.



virus when inoculated into the tomato simultaneously with either the "mottle" or "ringspot" viruses is in line with the findings of Ainsworth (1) and is a further indication of relationship. It does not, however, explain the fact that both "mottle" and "ringspot" viruses exist simultaneously in the same potato plant, as also may the "mottle" and "yellow mottle" viruses.

### Conclusion

The distinctive symptoms observed in the mosaic disease of President potatoes are due to the presence of a hitherto undescribed virus, which differs from both the "mottle" and the "ringspot" viruses in symptomatology upon test plants, especially in producing a yellow mottling on tomato and some other species. They also possess a number of points of similarity. All three viruses are able to infect the same range of host plants and can be separated from the "veinbanding" virus by passage through *Datura stramonium*. They have similar resistance to chemicals and have only slightly separated thermal death points. In combination with other viruses such as the "veinbanding" virus or tobacco virus 1 they cause similar symptoms on a number of host plants, especially in spot necrosis on nicotine or streak of tomato. The "yellow mottle" virus of President mosaic is therefore to be considered as an additional member of the "latent" or "X-virus" group.

### Acknowledgments

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## THE PARASITISM OF *CLADOSPORIUM FULVUM* COOKE AND THE GENETICS OF RESISTANCE TO IT<sup>1</sup>

BY ARTHUR N. LANGFORD<sup>2</sup>

### Abstract

Four physiologic forms of the imperfect fungus *Cladosporium fulvum* Cooke, causal agent of the leaf mold disease of tomatoes, have been differentiated by differences in pathogenicity. Cultural studies likewise have shown that this species is a composite of physiologic forms.

Saltant strains of differing degrees of stability have been isolated repeatedly from cultures arising from single one-celled spores. Since each cell of a conidium contains a single nucleus, these saltant strains are considered to arise as a result of mutations, in the broad sense of the term.

Four main classes of reaction to *C. fulvum* have been defined: complete susceptibility, two types of partial resistance, and immunity.

The reaction between pure lines of host and parasite is plastic. Environmentally conditioned variations in each of the four reaction types have been described. Of such variations the seasonal fluctuations in the reaction of *Lycopersicum esculentum* var. Stirling Castle to Form 1 are outstanding. It has been shown that the failure of the expression of the inherent resistance of this variety during midwinter at Toronto is due largely to the reduced light experience of plants grown at this time, while the failure of such plants to support sporulation is caused by the low relative humidity then prevalent in the greenhouses.

The genetics of the three types of resistance was fully analyzed. The Red Currant tomato, *L. pimpinellifolium*, carries, in addition to the dominant factor for immunity, an independently segregating dominant factor which, in the absence of the immunity factor, governs resistance to all four forms of *C. fulvum*. The resistance of Stirling Castle to Forms 1 and 3 has been shown to be due to another dominant factor.

Conspicuous among the genetic factors in the host which modify the main reaction types is the recessive lutescence factor in the homozygous condition. Its most striking effect is the production, on genetically immune individuals, of small inconspicuous infection spots whose increase in size is arrested very soon after symptoms appear.

As a result of linkage studies the three resistance factors have been located in MacArthur's (12) chromosome maps of the tomato.

The conflicting reports concerning the resistance of esculentum tomato varieties to *C. fulvum* are discussed in the light of physiologic specialization and of a plastic host reaction.

### Introduction

During the last decade, a voluminous literature has been built up around the leaf mold disease of tomatoes, but most of the reports have dealt with rather empirical methods of control. In many quarters, however, control through the use of resistant varieties has been sought, and Guba (9) has summarized the conflicting reports of fourteen investigators concerning the occurrence of resistance among ten of these varieties.

The present investigation began with a study of the linkage relations of the factor for immunity to *C. fulvum*, found in the Red Currant tomato. This led to the discovery of additional types of resistance and of physiologic specialization and to a study of the variability of *C. fulvum* and of the plasticity of host-parasite relations.

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Joint contribution from the University of Toronto, Departments of Botany and Biology and the Horticultural Experiment Station, Vineland Station, Ontario. Presented in May, 1936, to the University of Toronto in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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### Materials and Methods

In the early stages of the investigation, isolations from diseased plants were made by plating bits of infected leaf tissues which had been surface sterilized with mercuric chloride. After familiarity with the appearances of cultures of all ages was acquired, it was found safe and convenient to make direct transfers from fruiting lesions to agar slants, using a sterilized needle. Nearly all cultural studies were carried out on slants of potato-dextrose agar containing 2% dextrose and 2% agar.

Conidia for inoculation purposes were secured either from artificial cultures or from infected leaves. In the latter event the infections were produced by a strain which had been in culture previously and whose pathogenic capabilities were known. Conidial suspensions were made in tap water or in distilled water. Germination tests were commonly carried out in the same media, although it was shown that vigorous germination and growth occurred in normal decoctions (17) of the immune Red Currant tomato as well as in decoctions of resistant and susceptible esculentum varieties. It was observed frequently, in connection with germination tests, that spores capable of prompt and vigorous germination lost this ability after the suspension had been shaken for from five to ten minutes but regained it when the water was allowed to evaporate from the suspension, the spores germinating when again provided with conditions of high relative humidity. In general, vigorous plants from one to two months old, grown in four-inch pots, were used for inoculation purposes. In connection with studies of the inheritance of resistance, plants of the resistant variety were always included in the inoculations and  $F_1$  plants were frequently tested along with  $F_2$  plants. For actual inoculation the plants were removed from the greenhouse to avoid needless mixing of strains, and immediately after its preparation the suspension was applied to the lower surfaces of the leaves with a DeVilbiss atomizer. After the plants had stood for about an hour they were transferred to an inoculation chamber in which the relative humidity was maintained close to saturation. Inoculations were usually carried out in the evening and the plants were removed from the inoculation chamber the following evening.

When the securing of linkage data involved growing plants to maturity, e.g., for the observation of fruit colors, pathogenicity tests were carried out before the plants were transferred from the greenhouse to the field; in some instances further tests were carried out on cuttings from the plants in the field. Immer's (10) tables were used for the calculation of linkage intensities.

Material for the study of the nuclear situation in conidia was fixed for 24 hours in Bouin's fluid and stained with gentian violet according to Smith's (20) method.

### Experimental Results

#### STUDIES OF PHYSIOLOGIC SPECIALIZATION

The existence of physiologic forms of *C. fulvum* has been demonstrated by differences in the pathogenicity of various cultures. Tests involving 60 varieties revealed only one differential reaction, and of six esculentum varieties

that gave this reaction, Stirling Castle was chosen for subsequent studies. Stirling Castle is completely susceptible to some cultures of *C. fulvum* but, as a result of inoculation with others, gives a resistant reaction which, on young vigorous plants under conditions of abundant light and high relative humidity, is as follows. Symptoms appear in about the same time as on such susceptible varieties as Potentate but differ from them from the outset. The first whitish coloration due to the outward growth of fungous hyphae through the stomata of the leaf is restricted to a much smaller region than on Potentate. This restriction of development continues and, whereas in a few days infections on Potentate have spread considerably and turned brown in the centre owing to spore production (Plate I, Fig. 1, A), infections on Stirling Castle are not sporulating but appear as a compact whitish growth which is usually raised and puckered at the centre (Plate I, Fig. 1, B). At this stage infections on vigorous Potentate plants have not caused any conspicuous change in the appearance of the upper surface of the leaf opposite the infections, but those on Stirling Castle have caused a conspicuous yellowing of this surface. Whereas a heavy infection usually kills entire leaves on Potentate within a month after inoculation, comparable leaves on Stirling Castle are not killed but have, around the centres of infection, yellow spots which are more conspicuous on the upper than on the lower surface of the leaves. By this time many of these infections show a slight amount of sporulation, restricted to an area of from one to two millimetres in diameter at the centre of the spot.

In the pathogenicity tests to determine the extent of physiologic specialization 22 isolations of *C. fulvum* from 11 localities, one at Macdonald College, Quebec, the remainder in central and southwestern Ontario, were used. On Stirling Castle 15 of these produced the resistant reaction described above; the remaining 7 produced a reaction entirely comparable with the susceptible reaction on Potentate (Plate I, Fig. 2, B). Strains characterized by the production of a susceptible reaction on Potentate and of a resistant reaction on Stirling Castle are designated physiologic Form 1, and strains producing a susceptible reaction on both Potentate and Stirling Castle are designated physiologic Form 2 (Plate I, Fig. 2). Form 1 was obtained as follows: six times from Vineland Station, once from Macdonald College, Quebec, and once from the following localities in Ontario: Beamsville, Burlington, Colborne, Grimsby (two collections), Owen Sound, Simcoe and Vineland. Form 2 was obtained five times from Vineland Station and once from Beamsville and Vineland.

Since at Vineland Station, the only locality in which a number of collections were identified, five out of 11 cultures were of Form 2, it is believed that a study of a larger number of cultures would demonstrate a much wider distribution of this form than is indicated above.

Several preliminary inoculations indicated that symptoms differing from those produced by Forms 1 and 2 were in some cases the results of infections by mutant strains of the fungus. Accordingly a comprehensive inoculation

was made on September 4, 1934, under conditions of relative humidity enabling the development of sporulating infections. Vigorous uniform four-weeks-old plants of Potentate, Stirling Castle and five other varieties were inoculated in duplicate with 12 cultures of the fungus, including four known mutant strains which had been subcultured several times and six recently isolated cultures which had shown no evidence of mutation. The initial symptoms of infection on each variety were similar in the 12 series, but in some series, instead of the fungus growing out from the infected spots and sporulating, a progressive necrosis of the infected tissue occurred which, on susceptible varieties, resulted in enlarging dried-out spots resembling those following inoculation with "normal strains"<sup>\*</sup> of the fungus under conditions of low relative humidity (Plate II, Fig. 4). Reactions characteristic of Forms 1 and 2 were produced by five cultures, including four of those recently isolated. Three cultures, including two of the recent ones, produced a mixed reaction of sporulating and non-sporulating infections. The four mutant strains gave rise exclusively to necrotic non-sporulating infections. Stirling Castle was resistant to three of the latter and completely susceptible to the fourth. Strains characterized by the production of non-sporulating infections of a susceptible type on Potentate and similar infections of a resistant type on Stirling Castle are designated as physiologic Form 3 (Plate I, Fig. 3), whereas those producing similar infections of a susceptible type on both of these varieties are designated as physiologic Form 4 (Plate I, Fig. 4).

The similarity of the reactions produced by the mutant Forms 3 and 4 to those caused by normal strains under conditions of low relative humidity was strikingly demonstrated in a midsummer inoculation of six varieties with six cultures of single-spore origin. In all cases the infections were similar and without sporulation. That two types of reaction were involved, however, became evident when the plants were transferred to an inoculation chamber in which the relative humidity was maintained close to saturation, since in four of the six cases the fungus subsequently sporulated profusely while in the remaining cases it failed to sporulate.

The results of these pathogenicity tests have been supported by extensive cultural studies which indicate that the species *C. fulvum* is composed of an indefinite number of physiologic forms. Comparative studies of cultures obtained by isolating single one-celled conidia directly from sporulating infections have shown, on a single medium, differences in such characteristics as growth form and color. Also, as is shown below, many culturally distinct mutant strains have been isolated from a relatively small number of original cultures. It is not claimed that all mutant strains give rise to infections of the type produced by Forms 3 and 4. Furthermore, although the pathogenicity of a large number of original cultures has been tested it is quite possible that here, too, the existence of other physiologic forms may be demonstrated. The cultures used in this investigation were isolated from a comparatively

\* Any culture of the fungus which, under conditions of relatively high humidity such as have prevailed in our greenhouses in the spring and autumn of the year, produces sporulating infections on susceptible varieties is designated as a normal strain.

small area. Cultures from other localities may possess different parasitic capabilities and new differential hosts may be found. It seems likely, however, that the immunity of the Red Currant tomato, *L. pimpinellifolium*, will prove operative against all physiologic forms of the fungus, since, in the present investigation, it has shown itself immune from numerous cultures of the fungus and since other workers in widely separated centres (1, 4, 9, 18) have reported it immune from *C. fulvum*.

#### VARIABILITY OF *C. fulvum* IN CULTURE

Saltations occur frequently in cultures of *C. fulvum*, and the saltant strains, when isolated, exhibit great diversity in such cultural characteristics as growth rate, topography, color, amount of sporulation and the production of watery beads on the surfaces of the colonies.

Some idea of the variability of *Cladosporium* in culture may be obtained by following the behavior of the subcultures from a single spore, No. 51, which was isolated in August 1933, from a two-weeks-old culture from a natural infection. This spore produced on potato-dextrose agar a colony which, after a week's growth, was olive in color and of an irregular mounded contour. After 15 days a suspension of conidia from this colony was used in making cultures in Erlenmeyer flasks. In 11 days the surface of the agar in these flasks was covered with crowded colonies which had fruited abundantly, and at various places on their surfaces loose tufts of light colored mycelium had appeared. In March 1934, subcultures were made to 2% malt agar from one of these flask cultures and there resulted not only olive-colored colonies but also variants, two of which, one buff and one white, were isolated and found to be distinct in growth rate and colonial form as well as in color (Plate II, Fig. 1). The white and buff strains remained constant through five additional transfers extending to February 1935, whereas, from the olive strain during the same period, light-colored variants arose occasionally. The stability of the white and buff strains was further demonstrated in March 1935, when the three strains were passed through the host and recovered unaltered. Subsequently the light-colored strains have retained the same characteristics through five cultural generations on potato-dextrose agar. Two other variants from the olive strain from Spore 51 have been studied for more than a year; both have produced further variants. In February 1936, several saltants differing from any previously isolated were obtained from the same olive strain.

To discover whether the variability of cultures of *C. fulvum* was associated with a multinucleate condition of the conidia they were stained with gentian violet. The conidia were from one- to four-celled, with a single nucleus in each cell (Plate II, Fig. 5). It was evident, then, that the variability associated with a single nucleus could be determined from the study of the variability of cultures derived from single-celled spores. Accordingly, 20 single spores, 15 of them single-celled, were isolated directly from each of six sporulating infections. The original cultures resulting from the growth of these 120 spores showed a striking variability, patches of loose light-colored

mycelium appearing irregularly on practically every culture. The cultures from single-celled conidia were as variable as those from conidia having two cells. While the 20 individuals from each group were very similar in the types and frequency of saltations, differences in these respects were observed from group to group over a period of four months.

Of the original cultures 22 were selected for further study. When these cultures were five weeks old three subcultures were made from the olive-colored portion of each of them. After three months at least two of each group of three subcultures had produced variants. Attempts were made to make isolations from all the variants observed in these three-months-old subcultures, a small wisp of mycelium being picked off with a fine needle. Within two weeks these isolates exhibited marked differences in growth rate, production of watery beads, colonial form, and color, including whites, grays, buffs and olive greens. Triplicate cultures of the saltant strains were studied on a single medium and behaved uniformly. As many as seven distinct saltant strains were isolated from cultures resulting from a one-celled, uninucleate conidium.

The evidence presented above indicates that the variability of *C. fulvum* in culture is due to nuclear changes which are considered to be mutations, in the broad sense of the term, but whether they are due to gene mutations or chromosome aberrations cannot be stated.

#### THE PLASTICITY OF REACTION BETWEEN PURE LINES OF HOST AND PARASITE

In addition to those variations in the reaction of the tomato to *C. fulvum* which are due to the parasitic capabilities of genetically different strains of the parasite, and to those variations which are conditioned by genetic differences on the part of the host, and which will be treated in the section on the inheritance of resistance to *C. fulvum*, variations were observed when a pure line of the host was infected by a pure line of the parasite under various conditions. The influences on reaction type of environmental factors, of developmental stages, and of grafting will be considered.

##### *Environmental Factors*

The reaction of a tomato variety to *C. fulvum* has been shown repeatedly to depend on the general vigor of the inoculated plant. On vigorous plants of susceptible varieties little or no chlorosis is evident and the development of the fungus is vigorous, with abundant sporulation. On chlorotic plants and on plants whose growth has been appreciably retarded, infections cause a rapid chlorosis of the infected area, the distribution of the fungus is restricted and sporulation greatly reduced. No analysis has been made of the factors responsible for these variations. Schaffnit and Volk (16) and Volk (21), however, have demonstrated the dependence on nutrition, soil moisture, air moisture and other environmental factors of the expression of the leaf mold disease on completely susceptible varieties of tomato.

The reaction of the Red Currant tomato, *L. pimpinellifolium*, has likewise shown variations. It has been included in at least 100 inoculations carried out during every month of the year. In the vast majority of cases it has not shown macroscopic symptoms after inoculation. However, on five occasions, twice in May 1934, and in September 1934, October 1935, and November 1935, definite macroscopic evidence of infection has been observed on plants that were from four to five weeks old when inoculated. On November 6, 1935, eight days after inoculation with a strain which previously and subsequently failed repeatedly to induce symptoms on plants grown from the same seed lot, Red Currant plants showed minute brown necrotic flecks on the inoculated leaves. Bits of leaf tissue including these were surface-sterilized with 1 : 1000 mercuric chloride in water for from 1 to 1.5 minutes and plated out. *C. fulvum* was isolated in four out of eight cases, whereas four checks, bits of apparently uninjected portions of the inoculated leaves, failed to yield the fungus. The flecks did not increase in size and an attempt to isolate the fungus from them 21 days after inoculation failed. In May 1934 *C. fulvum* was isolated from similar infections on Red Currant 14 days after inoculation. In at least three other instances symptoms similar to the above have been observed in the *F<sub>2</sub>* from Red Currant  $\times$  esculentum, among individuals bearing the genetic factor for immunity that is found in Red Currant. It cannot be stated what environmental factors are responsible for the development of these symptoms on genetically immune individuals nor has it been possible to cause the production of such symptoms on other occasions.

Atypical symptoms of the disease have been observed also on 2R, a selection from the *F<sub>2</sub>* of the cross, Tangerine  $\times$  Red Currant; 2R has been tested repeatedly and is partially resistant to all known forms of the fungus, the lesions ordinarily failing to sporulate even after a long period of development (Plate III, Fig. 3). In September, 1934, however, infections produced on it by several strains of the fungus were sporulating moderately 15 days after inoculation. As in Red Currant the same strains produced typical infections on plants grown from the same seed lot when inoculations were made at a later date.

Temperature and humidity have been shown by Small (19) to exert a great influence on the development of various stages of the leaf mold disease. Thus, abundant sporulation was observed at 92% and 78% relative humidity, whereas at 58% the disease spots dried out and produced very few spores. The present investigation also showed this dependence of sporulation on relative humidity. When the relative humidity is low, typical sporulating infections are not produced on susceptible varieties by normal strains of the fungus; instead, the invaded leaf tissues are killed rapidly and a necrotic spot with a slowly advancing chlorotic margin results (Plate II, Fig. 4). All possible intergrades between infections of this type and the typical susceptible reaction have been observed. On various occasions plants having such necrotic non-sporulating lesions have been transferred to a large cloth inoculation chamber in which the relative humidity approached saturation.

In all these cases the fungus developed profusely at the margins of the lesions and sporulated within 24 hours. The type of development of infections in the inoculation chamber was the same as that which occurs initially on plants infected under humid conditions. Because of the rapidity with which normal development and sporulation was induced in these cases it is considered that the influence of relative humidity is largely upon the parasite.

The most striking instance of the plasticity of reaction type is the seasonal variation in the reaction of Stirling Castle to Form 1. In midwinter, instead of producing the resistant reaction (Plate I, Fig. 1, B) which has been described above, Stirling Castle reacts like susceptible varieties such as Potentate (Plate II, Fig. 4), except that the resulting spots are smaller (Plate II, Fig. 3).

A gradual change to the winter reaction and a gradual return, in the spring, to the summer reaction were indicated from the results of frequent inoculations carried out at Toronto over a period extending from October 1934 to April 1935. Since during this time length of day and light intensity fell to a minimum and subsequently rose again it seemed possible that light might be partly responsible for the observed variations in reaction. This was further suggested by preliminary experiments during the summer of 1935 in which the summer reaction was shifted slightly towards the winter type when the light experience of the plants had been reduced by covering them daily from noon until dusk with an aerated, black broadcloth cage (Plate II, Fig. 2).

The influence of light was studied in greater detail from weekly inoculations carried out at Toronto beginning October 8, 1935, at greenhouse temperatures well within the range of those enabling the development of reactions of a resistant type during the summer. Each week five- to seven-weeks-old plants of Potentate and Stirling Castle were inoculated with physiologic Form 1. With the approach of winter, in addition to the change in the light, the relative humidity of the greenhouse decreased and this induced a progressive decrease in sporulation. Thus, on Potentate, even the infections resulting from the first inoculation sporulated less than under conditions of high relative humidity. This decrease in sporulation was progressive until, with the inoculation of November 13, sporulation ceased. The influence of light, on the other hand, was evidenced by the amount of hypersensitiveness of the host.

On Stirling Castle, symptoms of essentially the normal summer type resulted from the inoculation of five-weeks-old plants on October 8. In successive inoculations there was a progressive but somewhat fluctuating increase in the size of the infection spots on Stirling Castle until, as a result of the inoculation of November 13, infections of the winter type described above were observed. At this time normal sporulation was observed on plants of both varieties that were covered each night by bell jars to increase the relative humidity.

Commencing December 5 the humidity of the whole greenhouse was greatly increased by placing on the heating pipes large pieces of burlap leading to water reservoirs. A relative humidity of 60%, as recorded on one greenhouse bench by a Julien P. Friez hygrothermograph, was maintained at temperatures

ranging from 70 to 80° F. After inoculation on December 18, symptoms became evident about the twelfth day and were very similar on the two varieties, the spots being diffuse, with a sparse mycelial growth on the under surface of the leaves. The restriction of development of the fungus which is observed on Stirling Castle plants at a comparable stage during the summer months was entirely lacking, and sporulation occurred on both varieties. After 24 days the heavily infected leaves on both varieties were almost completely killed and shrivelled, the necrosis of the infected tissues of Stirling Castle presenting a striking contrast to the chlorosis and lack of necrosis characterizing summer infections under comparable conditions.

On the basis of the results presented above it is considered that the seasonal variation in the reaction of Stirling Castle to physiologic Form 1, under the described range of environmental conditions, is attributable to a great extent to the effects of two factors, light and relative humidity. The reduced light experience of plants grown in midwinter is held to be largely responsible for the lack of expression of the genetic factor for resistance, while low relative humidity has been shown to be predominantly responsible for the lack of sporulation.

#### *Developmental Stages*

In the great majority of more than 150 inoculations, plants from one to two months old have been used and it has been observed repeatedly that symptoms appeared and developed later on the upper younger leaves than on the lower more mature ones. A regular gradation in time was observed but the type of reaction did not differ otherwise. On older plants with firm senile lower leaves a second gradation has often been observed. On the central and upper leaves of such Potentate plants normal infections developed, whereas the lower leaves displayed a progressive reduction in sporulation, the lowest showing only diffuse grayish spots which failed to sporulate even after a long period of development. The reaction of a leaf will thus be determined by its physiologic age.

#### *Grafting*

The reaction to *C. fulvum* of the components of grafts between *L. esculentum* and various solanaceous plants has been studied in considerable detail by Volk (21) and Bond (4). Bond has also studied the influence of grafting on the reaction of components of grafts between esculentum varieties known to differ in their reaction to *C. fulvum*. In the components of three graft combinations he found no change in reaction type due to grafting. Similar observations have been made during the present investigation. Following the inoculation of grafts between the esculentum varieties Potentate (completely susceptible) and Stirling Castle (resistant to physiologic Form 1), Potentate being used both as stock and as scion, no departure from the normal reactions for these varieties was observed.

STUDIES OF THE INHERITANCE OF RESISTANCE TO *C. fulvum**Host Range and Varietal Reaction*

Pathogenicity tests have been restricted to species of the genus *Lycopersicum*, and to *Solanum melongena*, the only other species reported susceptible to this fungus (7). Young plants of the eggplant varieties Black Beauty and Blackie were found to be immune from strains of the fungus that produced typical symptoms of leaf mold on susceptible varieties of tomato inoculated at the same time. Tests have been made of the reaction of 51 *L. esculentum* varieties which included both old and modern commercial types as well as small-fruited varieties; of *L. Humboldtii* from two sources; of *L. pimpinellifolium* from several sources; of hybrids between *L. esculentum* and *L. pimpinellifolium*; and of two wild tomatoes whose relationships to these species are uncertain. Four distinct types of reaction to normal strains of *C. fulvum*, each of which may be modified by environmental factors or by genetic factors in the host, were revealed by these tests:

1. *Potentate*. Potentate is completely susceptible to all known physiologic forms of the fungus. On it are produced the normal symptoms of the disease (Plate I, Fig. 1, A; Plate III, Fig. 1). Other completely susceptible varieties are: *L. esculentum* varieties Acme, Ailsa Craig, Ailsa Craig green stem mutant  $a_2$ , Banalbufar, Beauty, Bonny Best, Break O'Day, Buckeye State, Burbank Preserving, Chalk's Early Jewel, Cooper's Special, Crackerjack, Denton's Special, Devon Surprise, Dwarf Aristocrat, Dwarf Stone, Earliana, Early Detroit Purple, First of All, Golden Queen, Golden Dwarf Champion, Grand Rapids, Grape Cluster, Honor Bright, Imperial Globe, King Humbert, Marglobe, McMullen Pink, Norton, Oxheart, Peach, Ponderosa, Pritchard, Red Cherry green stem mutant  $a_1$ , Red Pear, Rouge Naine Hative, Rough Trophy, Satisfaction, Stone, Sunrise, Tangerine, Wonder of Italy, Yellow Pear, Yellow Plum and five selections made by J. W. MacArthur; selections of *L. Humboldtii* from Copenhagen and Frankfurt; a hybrid received as *L. pimpinellifolium* fructo luteo and wild tomatoes from Jamaica and Mexico.

2. *Stirling Castle*. This variety is completely susceptible to physiologic Forms 2 and 4 but resistant to Forms 1 and 3. The Stirling Castle type of reaction (p. 110; Plate I; Plate III, Fig. 2) occurred in the esculentum varieties Best of All, Maincrop, Norton's Wilt-resistant Stone (not homozygous), Tuckswood Favourite and Up-to-date.

3. *2R selection*. This selection (p. 114) is resistant to all known forms of the fungus. Under conditions of abundant light and relatively high humidity, symptoms appear in about the same time as on Potentate and Stirling Castle but unless the relative humidity is very high the fungus does not grow out through the stomata to an appreciable extent, even after a long period of development. The infected tissue becomes chlorotic and the resulting spots are similar in appearance on both surfaces of the leaf (Plate III, Fig. 3). Necrosis is not evident by the time infections on comparable Potentate plants are sporulating profusely, but usually some necrosis occurs eventually, its

extent varying from plant to plant. The single instance in which sporulating infections were observed on 2R has been described above. Three sixteenths of the  $F_2$  individuals of crosses between *L. pimpinellifolium* and completely susceptible varieties react similarly to 2R.

4. *Red Currant*. Although this species of tomato is ordinarily considered immune from *C. fulvum* and macroscopic symptoms do not typically follow inoculation (Plate III, Fig. 4), bits of leaf tissue cleared and stained in lactophenol with cotton blue show that the fungus penetrates the plant through the stomata and develops in the leaf tissue to a very limited extent. Furthermore, in five instances among more than 100 inoculations infection flecks have been observed on Red Currant (p. 114). The immunity reaction was observed only in *L. pimpinellifolium* and in derivatives from it.

#### Mendelian Nature of the Factors Governing Resistance

##### The Two Resistance Factors of the Red Currant Tomato

Tests of entire  $F_2$  populations of crosses between the immune Red Currant tomato and typically susceptible esculentum varieties have been carried out to discover whether a linkage exists between the factor for immunity and some factor or factors included in MacArthur's (12) chromosome maps. In addition to the parental types a third class of individuals, partially resistant to all known forms of the fungus, was observed in such populations. Among the latter there was considerable variation, even under the same environmental conditions, and it was often difficult to classify the individuals at the extremes of this range. Under conditions of relative humidity enabling the development of sporulating infections on Potentate the majority produced non-sporulating infection spots similar to those described for 2R selection. Under very humid conditions some individuals sporulated but they could be distinguished from typically susceptible individuals on which the spots spread over a greater area. Resistant individuals of this type have been found in all  $F_2$  populations of Red Currant crosses but never among esculentum varieties.

The genetic basis of these results is elucidated by the data secured from the inoculation, in the summer of 1934, of 500  $F_2$  individuals of a cross, Red Currant  $\times$  esculentum. Because of the wide range of symptoms resulting it was necessary to inoculate some of the plants several times before a conclusive classification could be made. The final observed ratio was 379 immune : 90 partially resistant : 31 typically susceptible. This conforms very closely to a 12 : 3 : 1 ratio which is 375 : 93.8 : 31.2 for a population of 500. The results secured in this instance have been supported by those obtained from all other  $F_2$  populations. They are explained on the basis that *L. pimpinellifolium* carries, in addition to the dominant factor for immunity, an independently segregating dominant factor for resistance which is expressed only in the absence of the factor for immunity, i.e., is hypostatic to it. The symbol  $Cf_{p1}$  is assigned to the factor governing immunity and the symbol  $Cf_{p2}$  to the resistance factor. The single factor basis of these

two resistances and the dominance of the two factors are confirmed by back-cross data. For instance, the inoculation of 178 plants from three populations yielded the ratio 97 immune : 44 resistant : 37 susceptible; the deviations from theoretical 1 : 1 ratios are 1.2 and 0.7 times the standard error for  $Cf_{p1}$  and  $Cf_{p2}$ , respectively.

The factor  $Cf_{p1}$  behaves as a complete dominant, even in the presence of a chromosome complex derived largely from typically susceptible esculentum varieties. On the other hand, there is considerable evidence that the  $Cf_{p2}$  factor is not fully dominant. In some inoculations it seemed possible to divide the resistant individuals into heterozygotes and homozygotes; the suspected homozygotes displayed infections resembling those on 2R selection, which is known to be homozygous for  $Cf_{p2}$ , and the suspected heterozygotes showed larger infections with necrosis or some sporulation. However, this possibility was not tested by the growing of  $F_3$  populations.

Conspicuous among the factors modifying the expression of the main resistance factors is the lutescence factor which in the homozygous condition not only determines lutescence but also modifies the expression of the  $Cf_{p1}$  factor. On lutescent, genetically immune individuals from crosses between Red Currant and esculentum varieties there appear, following inoculation, small conspicuous chlorotic spots the centres of which rapidly become necrotic. These spots are much more conspicuous than the flecks that have been observed a few times on non-lutescent, genetically immune individuals, but like them do not increase noticeably in size, their maximum diameter being less than 2 mm. (Plate II, Fig. 6). The contrasting reaction of a partially resistant, lutescent hybrid is shown in Plate II, Fig. 7.

#### *The Resistance Factor of Stirling Castle*

The variety Stirling Castle has long been recognized as somewhat resistant to *C. fulvum* (11). This resistance has been described and it has been noted that Stirling Castle is resistant to Forms 1 and 3 but completely susceptible to Forms 2 and 4. While the following results are based on reactions to Form 1 it is believed that similar results would be obtained with the closely related Form 3.

The reaction of  $F_1$  individuals from crosses between Stirling Castle and seven completely susceptible varieties (Burbank Preserving, Potentate, Rouge Naine Hative, a wild tomato from Mexico and three esculentum selections) was tested on several occasions during the summer months. In all cases their reaction was very nearly the same as that of comparable Stirling Castle plants, thus demonstrating that under the prevailing environmental conditions the resistance of Stirling Castle is dominant.

From nine inoculations of a total of 773  $F_2$  individuals from five such crosses the observed ratio was 557 resistant : 216 susceptible, which deviates from a 3 : 1 ratio by 1.8 times the standard error. The resistance of Stirling Castle, therefore, is governed by a single dominant Mendelian factor. The symbol  $Cf_{sc}$  is assigned to this factor, the distinctness of which from  $Cf_{p2}$  is evident

from the fact that the latter confers resistance to all known forms of the fungus, whereas  $Cf_{sc}$  does not confer a resistance to Forms 2 and 4, and also from the fact that an  $F_2$  of the cross, 2R selection  $\times$  Stirling Castle, yielded completely susceptible individuals as well as parental types. The degree of dominance of the  $Cf_{sc}$  factor has been shown to vary with varying environmental conditions. In a number of the inoculations the resistant and susceptible reactions were so clearly defined that there could be no possible doubt as to the classification of an individual. In June 1935, however, amongst a population of 215  $F_2$  individuals, a wide range of reactions was observed, extending from typical resistant to typical susceptible types. The amount of sporulation ranged from profuse on typically susceptible individuals, through very slight on individuals which certainly carried one  $Cf_{sc}$  factor, to none at all on other individuals. A few individuals could not with certainty be classified as resistant or susceptible. The fact that more than two types of reaction were observed demonstrates the incompleteness of the dominance of the resistance factor under the conditions of the experiment, whereas the wide range of reactions among genetically resistant individuals indicates the presence of modifying factors. These variations are probably attributable in large measure to very high relative humidity and to the extremely rapid growth of the plants. This explanation seems more likely in view of the fact that a clearly defined segregation of resistant and susceptible individuals resulted from the inoculation, five days later, of smaller less vigorous plants of the same  $F_2$  population. It is also pointed out that under conditions enabling the expression of  $Cf_{sc}$  as a nearly complete dominant the effect of modifying factors is scarcely apparent, the range of symptoms being very narrow.

When backcross populations of Stirling Castle crosses were inoculated in June and July 1935, a sharp segregation of resistant and susceptible individuals resulted. The observed ratio of 194 resistant : 179 susceptible, which deviates from a 1 : 1 ratio by 0.9 times the standard error, demonstrates further the single factor basis of the resistance of Stirling Castle. In these populations some of the resistant individuals supported slight to moderate sporulation; as all the resistant individuals were heterozygous for  $Cf_{sc}$ , this variation in the amount of sporulation is a clear demonstration of the presence of modifiers. In those instances in which Stirling Castle was crossed with a lutescent parent the recessive factor governing lutescence was outstanding as a modifier of reaction type. On completely susceptible individuals lutescence caused a more pronounced gradation from sporulating to non-sporulating infections than has been observed on plants with senile leaves. In many cases this gradation was pronounced, even on single leaves intermediate in position between leaves with and without sporulation; here, terminal leaflets showed no sporulation while infections on the younger lateral leaflets were sporulating profusely. On both resistant and susceptible individuals lutescence causes a shortening of the incubation period of *C. fulvum*, symptoms regularly appearing from one to two days earlier than on normal non-lutescent individuals. In addition,

the infections on lutescent individuals are of a bright yellow color and are strikingly conspicuous on those upper leaves which are still green. A separation of hybrid populations into lutescent and normal individuals can be made readily on the basis of these differential reactions.

#### *Linkage Relations of the Factors Governing Resistance*

Linkage tests applied to the three resistance factors involved 19 pairs of characters whose monofactorial inheritance has been proved by MacArthur(12) and Butler (5). Data were secured from a study of  $F_2$  and of backcross populations of crosses made in the coupling phase, *i.e.*, both dominants entered the cross from one parent and both recessives from the other.

#### *Cf<sub>p1</sub>, the Factor for Immunity*

The linkage relations of this factor with 15 others distributed on nine chromosomes are summarized in Table I. It will be seen that  $Cf_{p1}$  is situated

TABLE I  
LINKAGE DATA FOR  $Cf_{p1}$ , THE FACTOR FOR IMMUNITY, SECURED FROM  $F_2$  POPULATIONS

Factor pair tested	Chromosome number on which located	Segregation ratios*				Number of plants tested	Percentage crossing over with standard error	Deviation in terms of the standard error
		X Y	X y	x Y	xy			
$D_1 d_1$	I	893	223	285	71	1472	50.0 ± 1.9	0.0
$P_1 p$	I	301	77	97	24	499	50.5 ± 3.4	0.1
$R_1 r$	II	282	97	97	24	500	54.6 ± 3.1	1.5
$Y_1 y$	III	382	108	122	37	649	49.0 ± 3.0	0.3
$C_1 c$	IV	787	217	214	98	1316	43.0 ± 1.9	3.7
$F_1 f$	V	302	77	92	29	500	47.0 ± 2.8	1.1
$A_1 a_1$	V	529	181	162	64	936	47.8 ± 2.4	0.9
$L_1 lf$	V	306	73	87	34	500	43.2 ± 2.8	2.4
$J_1 j$	V	304	75	86	35	500	43.1 ± 2.8	2.5
$L_1 l$	VI	728	231	228	63	1250	51.5 ± 2.2	0.7
$U_1 u$	VII	82	29	28	10	149	49.9 ± 6.1	0.0
$H_1 h$	VII	1374	404	427	144	2349	48.1 ± 1.5	1.3
$T_1 t$	VII	82	29	28	10	149	49.9 ± 6.1	0.0
$A_2 a_2$	VIII	125	32	55	10	222	54.8 ± 5.3	1.0
$Wt_1 wt$	X	323	56	106	15	500	52.8 ± 3.4	2.1

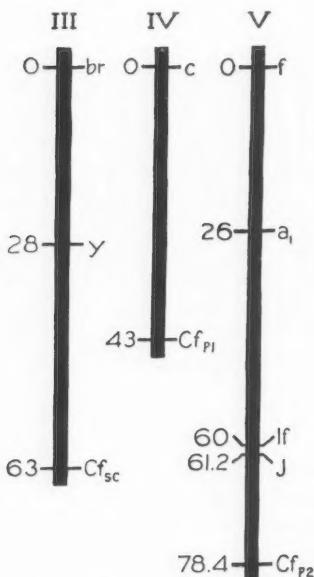
\* In this and in subsequent tables:

X = the dominant and x = the recessive of the factor pair governing resistance and susceptibility.

Y = the dominant and y = the recessive of the other factor pair tested.

on chromosome IV, loosely linked with  $c$ , the factor for potato leaf (Text-fig. 1). The position of this immunity factor on the fourth chromosome cannot be stated more accurately until an examination is made of its linkage relations with  $sp$ , the factor for self-pruning habit of growth, which is very closely linked with  $c$ . Tests were also carried out to determine whether there was any linkage between the immunity factor and factors determining fruit size. For this purpose 534  $F_2$  plants of two Red Currant  $\times$  esculentum crosses

were grown to maturity in the field. Mean fruit weights were determined from the averages of 20 ripe fruits per plant and the deviations expressed in terms of the standard error. In one population, comprising 384 plants, the mean fruit weights of  $Cf_{p1}$  and  $cf_{p1}$  individuals were  $7.2 \pm 0.1$  gm. and  $7.3 \pm 0.4$  gm. respectively and the insignificant difference between the two classes  $0.1 \pm 0.4$  gm. From a second population the corresponding figures were  $10.0 \pm 0.4$  gm. and  $10.4 \pm 1.0$  gm. with a difference of  $0.4 \pm 1.2$  gm., again not a significant one. Although these data show no indication of a linkage between the immunity factor and fruit size factors, an article by MacArthur and Butler (13) indicates that such a linkage might be discovered in populations from other crosses.



TEXT-FIG. 1. Chromosomes III, IV, and V, showing loci of  $Cf_{sc}$ , the resistance factor from Stirling Castle,  $Cf_{p1}$ , the immunity factor from the Red Currant tomato, and  $Cf_{p2}$ , the resistance factor from the Red Currant tomato.

#### $Cf_{p2}$ , the Resistance Factor from Red Currant

Since the linkage data involving this factor were secured from the same populations that furnished the data for the immunity factor, only about one-fourth as many plants are included in the ratios observed. The linkage relations of the resistance factor are summarized in Table II, from which it is seen that  $Cf_{p2}$  is located on chromosome V, linked fairly

TABLE II  
LINKAGE DATA FOR  $Cf_{p2}$ , THE RESISTANCE FACTOR FROM RED CURRANT, SECURED FROM  $F_2$  POPULATIONS

Factor pair tested	Chromosome number on which located	Segregation ratios				Number of plants tested	Percentage crossing over with standard error	Deviation in terms of the standard error
		X Y	X y	x Y	xy			
$D_1 d_1$	I	24	5	13	2	44	$54.3 \pm 11.8$	0.4
$P p$	I	72	18	25	5	121	$50.6 \pm 6.8$	0.0
$R r$	II	73	17	24	7	121	$46.8 \pm 6.5$	0.5
$Y y$	III	94	27	28	10	159	$47.0 \pm 5.8$	0.5
$C c$	IV	22	7	10	5	44	$43.7 \pm 10.5$	0.6
$F f$	V	71	19	21	10	121	$42.0 \pm 6.2$	1.3
$A_1 a_1$	V	24	5	11	4	44	$42.3 \pm 10.4$	0.7
$Lf lf$	V	78	12	9	22	121	$18.4 \pm 4.0$	7.9
$J j$	V	78	12	8	23	121	$17.2 \pm 3.8$	8.6
$U u$	VII	23	8	5	2	38	$48.0 \pm 11.8$	0.2
$H h$	VII	117	33	36	17	203	$42.9 \pm 4.9$	1.4
$T t$	VII	22	9	6	1	38	$>60 \pm 10.4$	1.0

closely with *j* and *lf*, factors for jointless pedicel and leafy inflorescence respectively. The locus of the resistance factor on chromosome V is plotted in Text-fig. 1.

#### *Cf<sub>sc</sub>*, the Resistance Factor from Stirling Castle

Table III summarizes the linkage relations of this factor with 17 others distributed on 10 chromosomes. It is seen that *Cf<sub>sc</sub>* is linked with the *Yy* factor pair, which governs the character contrasts yellow *vs.* clear fruit epicarp.

TABLE III  
LINKAGE DATA FOR *Cf<sub>sc</sub>*, THE RESISTANCE FACTOR FROM STIRLING CASTLE

Factor pair tested	Chromosome number on which located	Segregation ratios				Number of plants tested	Percentage crossing over with standard error	Deviation in terms of the standard error
		<i>X</i> <i>Y</i>	<i>X</i> <i>y</i>	<i>x</i> <i>Y</i>	<i>xy</i>			
<i>F</i> <sub>2</sub> data:								
<i>D</i> <sub>1</sub> <i>d</i> <sub>1</sub>	I	135	41	66	23	265	48.1 ± 4.4	0.4
<i>P</i> <i>p</i>	I	135	40	64	24	263	49.9 ± 4.6	0.0
<i>O</i> <i>o</i>	I	115	60	56	32	263	48.7 ± 4.6	0.3
<i>S</i> <i>s</i>	I	135	40	68	20	263	51.1 ± 4.7	0.2
<i>R</i> <i>r</i>	II	137	38	70	16	261	51.7 ± 4.7	0.4
<i>Y</i> <i>y</i>	III	148	27	48	38	261	30.9 ± 3.6	5.3
Backcross data:								
<i>D</i> <sub>1</sub> <i>d</i> <sub>1</sub>	I	90	104	99	80	373	54.4 ± 2.5	1.8
<i>R</i> <i>r</i>	II	59	42	34	52	187	40.7 ± 3.6	2.6
<i>Br</i> <i>br</i>	III	45	48	46	47	186	50.5 ± 3.7	0.1
<i>Y</i> <i>y</i>	III	61	40	35	51	187	40.1 ± 3.6	2.8
<i>C</i> <i>c</i>	IV	59	42	45	41	187	46.5 ± 3.7	0.9
<i>F</i> <i>f</i>	V	50	43	53	39	185	51.9 ± 3.7	2.2
<i>A</i> <sub>1</sub> <i>a</i> <sub>1</sub>	V	56	45	48	38	187	49.7 ± 3.7	0.1
<i>Lf</i> <i>lf</i>	V	36	57	42	50	185	53.5 ± 3.7	0.9
<i>J</i> <i>j</i>	V	36	57	43	49	185	54.1 ± 3.7	1.6
<i>L</i> <i>l</i>	VI	54	47	34	52	187	42.3 ± 3.6	2.1
<i>H</i> <i>h</i>	VII	50	43	50	42	185	50.3 ± 3.7	0.1
<i>A</i> <sub>2</sub> <i>a</i> <sub>2</sub>	VIII	47	46	42	50	185	47.6 ± 3.7	0.6
<i>D</i> <sub>2</sub> <i>d</i> <sub>2</sub>	IX	46	47	45	47	185	49.7 ± 3.7	0.1
<i>Wt</i> <i>wt</i>	X	53	40	42	50	185	44.3 ± 3.7	1.5

Whereas the backcross gives a crossover value between these factors of 40.1%, the *F*<sub>2</sub> gives the value 30.9%. Since these two measures are equally significant the true crossover value between *Cf<sub>sc</sub>* and *y* is approximately 35% (Text-fig. 1).

#### Discussion

Variant strains of *C. fulvum* have heretofore been reported only by Caldis and Coons (6), who considered that they were Dauermodifikationen. They did not, however, present acceptable evidence of their reversion to the parental form, nor have the present studies of the variability of *C. fulvum* revealed a single instance of even apparent reversion. Moreover, variants have appeared

suddenly, at irregular intervals and in localized positions haphazardly disposed, and although many differ but slightly from parental forms, other variants are so distinct that they might not be recognized as *C. fulvum*. In view of these facts and since many distinct variants may be isolated from the product of growth from a single-celled uninucleate conidium it is considered that they arise as a result of mutations, in the broad sense of the term outlined by Dickinson (8).

Since *C. fulvum* is propagated asexually, no perfect stage having been reported throughout its wide geographic range, it is interesting to speculate as to what extent the variability of the fungus in culture is characteristic of its behavior in nature. The existence of a number of physiologic forms in nature has been indicated by pathogenicity tests and by observations of original cultures and, unless we assume the extreme position that the constitution of the species has been static for many years, the most reasonable explanation of the occurrence of physiologic forms is that they arise as a result of mutations.

Only one light-colored form has been isolated directly from nature in about 300 attempts, whereas such forms compose a large percentage of the mutants isolated from artificial cultures. This may be due in part to the failure of some of these forms to persist, inasmuch as inhibition or reduction of sporulation has characterized many light-colored mutants. Another possible explanation arises from our pathogenicity tests which, though limited in number, indicate that many mutant strains, including some which sporulate freely in culture, are unable to sporulate on the host. On the other hand it is conceivable that the stimuli responsible for their frequent occurrence in culture do not operate to the same extent in nature.

Whether the mutations in *C. fulvum* are chromosomal aberrations or gene mutations or whether both these types of hereditary changes occur, cannot be stated. The possibility of arriving at a decision on this question from a study of hybrids is precluded by the absence of a perfect stage. *C. fulvum* possesses, however, many qualities which make it valuable for a study of variation. Pure lines may be obtained readily, the growth of the fungus in culture is relatively slow, mutations occur frequently and many of them are striking in cultural characteristics. Furthermore, distinct qualitative differences in the symptoms of disease are observed and some mutants show changes in pathogenicity. Thus, it is considered that further studies of the variability of *C. fulvum* may add materially to our knowledge of the mechanisms of variation in the Fungi Imperfecti.

The reaction of the tomato to *C. fulvum* is very plastic. Schaffnit and Volk (16), Volk (21) and Small (19) have carried out careful experiments that demonstrate the dependence of the reaction of completely susceptible varieties on various environmental factors. There are, however, no reports in the literature concerning the modification of the reaction of resistant varieties. In the present investigation it has not been possible to relate the rare and irregular occurrence of symptoms of infection on the typically immune

Red Currant tomato to the effect of specific environmental factors. On the other hand, the seasonal variation in the reaction of the variety Stirling Castle to physiologic Form 1 under our experimental conditions has been shown to be due in large part to the influences of relative humidity and light. The demonstration that the failure of the fungus to sporulate on diseased plants during the winter months is due to low relative humidity is in accord with the results of Small (19) and Volk (21). Although a definite relation between reduction of the light experience of plants and the modification of their reaction in the direction of susceptibility has been revealed, our experiments are to be regarded as preliminary ones. Such factors as soil type, nutrition, soil moisture and age of plants were not well controlled and from the work of Schaffnit and Volk (16) and Volk (21) it is apparent that these and other factors must be controlled if uniform experimental material is to be available. Our results do not suggest the manner in which light is operative, but the solution of this striking instance of the dependence of the expression of a known genetic factor on environmental conditions would be a valuable contribution to our knowledge of the nature of disease resistance.

The necessity of employing, when possible, distinct qualitative differences in host reaction for the estimation of resistance and susceptibility to fungi has been illustrated during the course of the present investigation. Von Sengbusch and Loschakowa-Hasenbusch (18) failed to make a complete separation of types of reaction to *C. fulvum* in their study of  $F_2$  populations of the general cross, Red Currant  $\times$  completely susceptible esculentum varieties, and thus reported a segregation ratio of 3 immune : 1 susceptible for such populations. In our tests of similar  $F_2$  populations a ratio of 12 immune : 3 partially resistant : 1 completely susceptible has been observed and thus an additional type of resistance has been revealed.

Guba (9) has summarized the conflicting reports of fourteen investigators concerning the occurrence of resistance to *C. fulvum* among the esculentum varieties Frogmore Selected, Lucullus, Norton, Satisfaction, Stirling Castle, Stone, Tuckswood and Up-to-date. There is similar disagreement concerning the inheritance of resistance in these varieties: the resistance of Stirling Castle is reported both dominant (19) and recessive (2, 18), as is that of Satisfaction (2, 15), while resistance is reported recessive in Maincrop (15). Small's  $F_1$  data (19) indicate that the resistance of Up-to-date is dominant, yet a later report (3) states that the  $F_2$  of certain crosses with Up-to-date yielded only susceptible individuals while other crosses with this variety yielded both resistant and susceptible individuals. Our studies of physiologic specialization and of the plasticity of host-parasite relations are significant in relation to the data just given. The variety Stirling Castle is resistant to physiologic Forms 1 and 3 but completely susceptible to Forms 2 and 4. Although Makemson (14), Caldis and Coons (6) and Bond (4) isolated single spores of *C. fulvum*, other workers have used spores from natural infections for inoculation purposes, and it is considered that the failure to relate the results secured on different occasions to specific physiologic forms may explain many of the

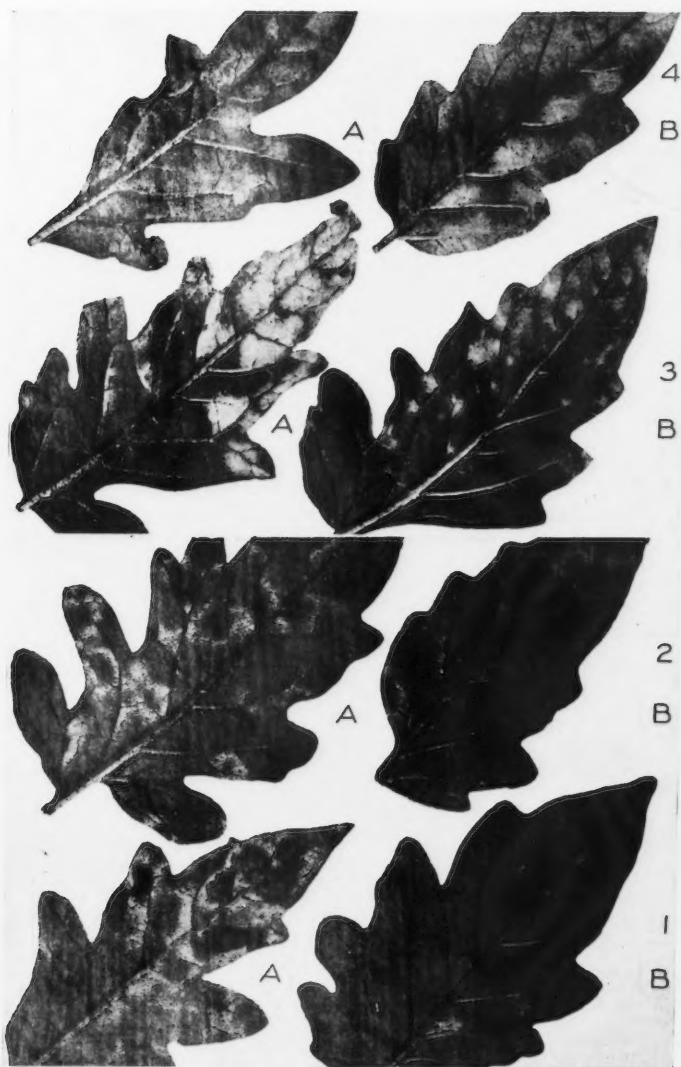
contradictions outlined above. In connection with resistance studies a recognition of the plasticity of the reaction between pure lines of host and parasite is equal in importance to that of the existence of physiologic forms of *C. fulvum*. It has been shown that the degree of dominance of the genetic factor governing the resistance of Stirling Castle may vary with varying environmental conditions over a brief space of time and it is suggested that this circumstance may have led to some confusion in inheritance studies. More important, however, are the seasonal variations in the reaction of Stirling Castle, whose inherent resistance is but slightly expressed during midwinter, at Toronto. Varieties resistant during the summer would be classified as susceptible during midwinter. It may readily be seen that an exact assessment cannot be made of the responsibility of each of the factors just considered for the contradictory nature of the available data, but it is clear that these data concerning the resistance of esculentum varieties to *C. fulvum* must be reanalyzed. Although our studies have not revealed any differences among six such resistant varieties it is possible that these may be differentiated through the discovery of other physiologic forms of the fungus. In connection with a reanalysis of the resistance problem it is essential that the reaction of hybrids and of parents be tested simultaneously and that the environmental conditions under which a variety is resistant, as well as the relation of this resistance to physiologic forms of the fungus, be defined clearly.

The dependence of the expression of those main factors governing resistance to *C. fulvum* on other genetic factors in the host, as well as on environmental conditions, has been shown clearly. Modifiers of the factors governing the two partial resistances have been demonstrated repeatedly from the behavior of  $F_2$  and of backcross populations. The only modifier that has been recognized apart from its effect on host reaction is that governing the production of lutescent foliage. In contrast to the two resistance factors, the immunity factor of the Red Currant tomato has been singularly independent in its effect, except in the presence of two of the recessive factors governing lutescence. This independence of the factor governing immunity is of great practical significance from the standpoint of developing commercially desirable immune varieties. During the last three years lines carrying this factor have been backcrossed repeatedly to completely susceptible commercial varieties of tomatoes and it has been found that, even in the presence of a chromosome complex derived largely from esculentum varieties, the expression of this immunity factor is not modified.

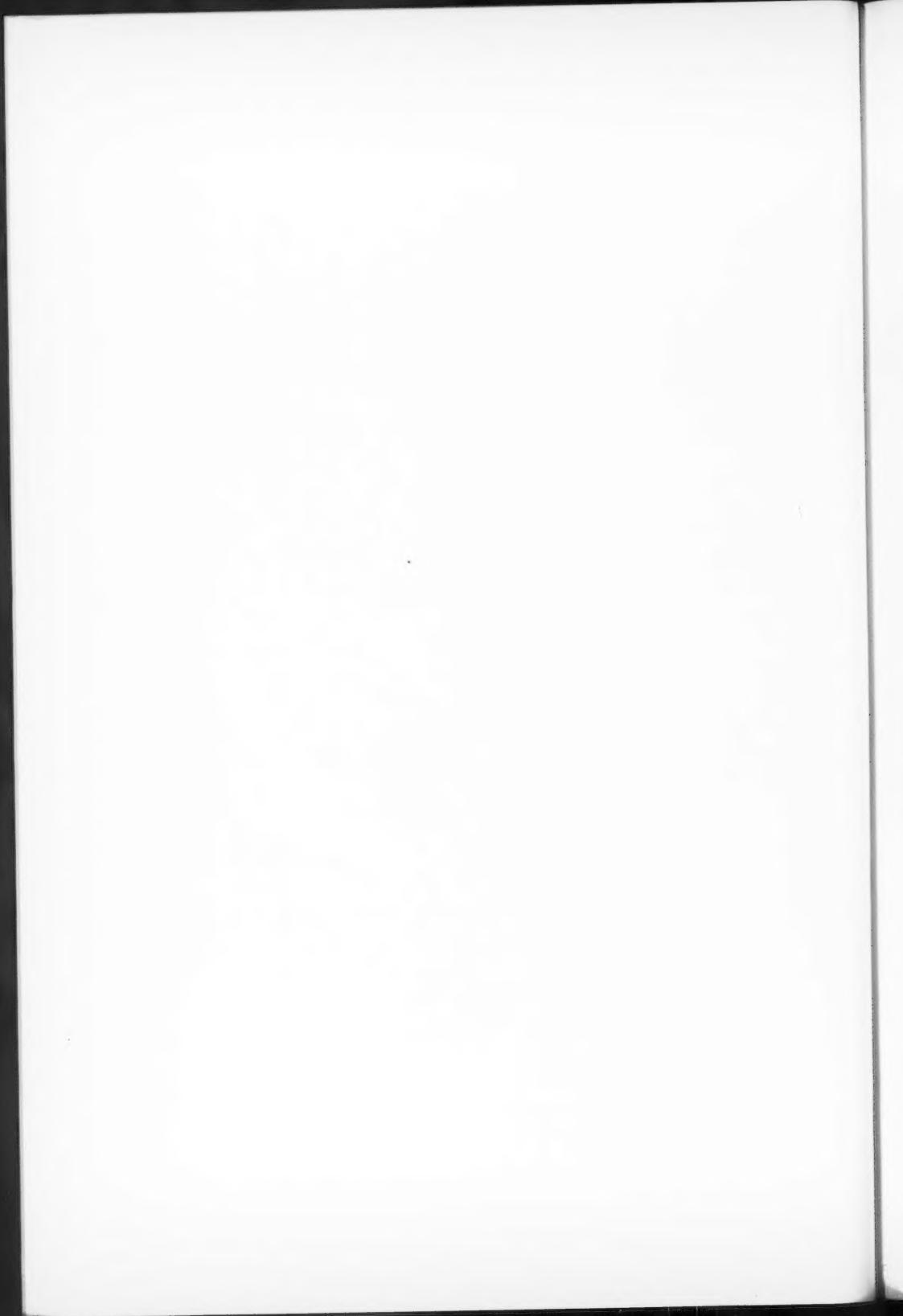
The present studies of varietal reaction and of the inheritance of resistance to *C. fulvum* have involved three types of resistance. The immunity of the Red Currant tomato and the genetic basis of this immunity had been reported already by other workers (18) but the resistance of Stirling Castle has been defined clearly for the first time and a third type of resistance (also found in the Red Currant tomato) has been discovered.

To represent the dominant factors governing these three resistances, universally significant symbols were selected, which would not lead to con-

PLATE I



Figs. 1, 2, 3 AND 4. Reactions of Potentate (A) and Stirling Castle (B) to physiologic Forms 1, 2, 3 and 4, respectively, of *C. fulvum*. Lower leaf surfaces 16 days after the inoculation of 29-days-old plants in September 1934, under conditions of high relative humidity.  $\times 1$ .



## PLATE II

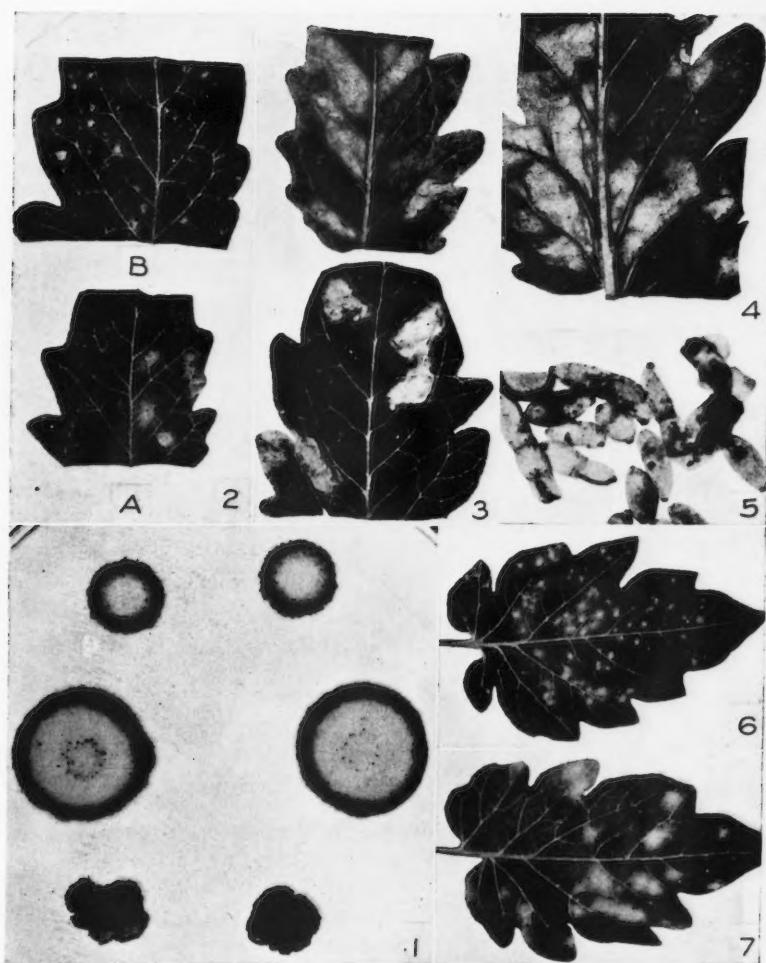


FIG. 1. Three strains from culture No. 51 of *C. fulvum* after one month's growth on 2% malt agar. FIGS. 2 AND 3. Variations in the reaction of Stirling Castle to physiologic Form 1. FIG. 2. Upper leaf surfaces 19 days after the inoculation, in summer, of 38-days-old plants: A. normal plant, B. darkened plant. FIG. 3. Symptoms 27 days after inoculation of ten-weeks-old plants in November; relative humidity low. FIG. 4. Lower leaf surface of Potentate: other dates as for Fig. 3. FIG. 5. Conidia of *C. fulvum* from natural infections, stained with gentian violet ( $\times 600$ ). FIGS. 6 AND 7. Reaction of lutescent individuals to Form 2, 17 days after August inoculation of month-old plants; upper leaf surfaces. FIG. 6. A genetically immune Red Currant hybrid. FIG. 7. A hybrid carrying the factor for partial resistance to all forms of *C. fulvum*. All photographs except Fig. 5 are natural size.

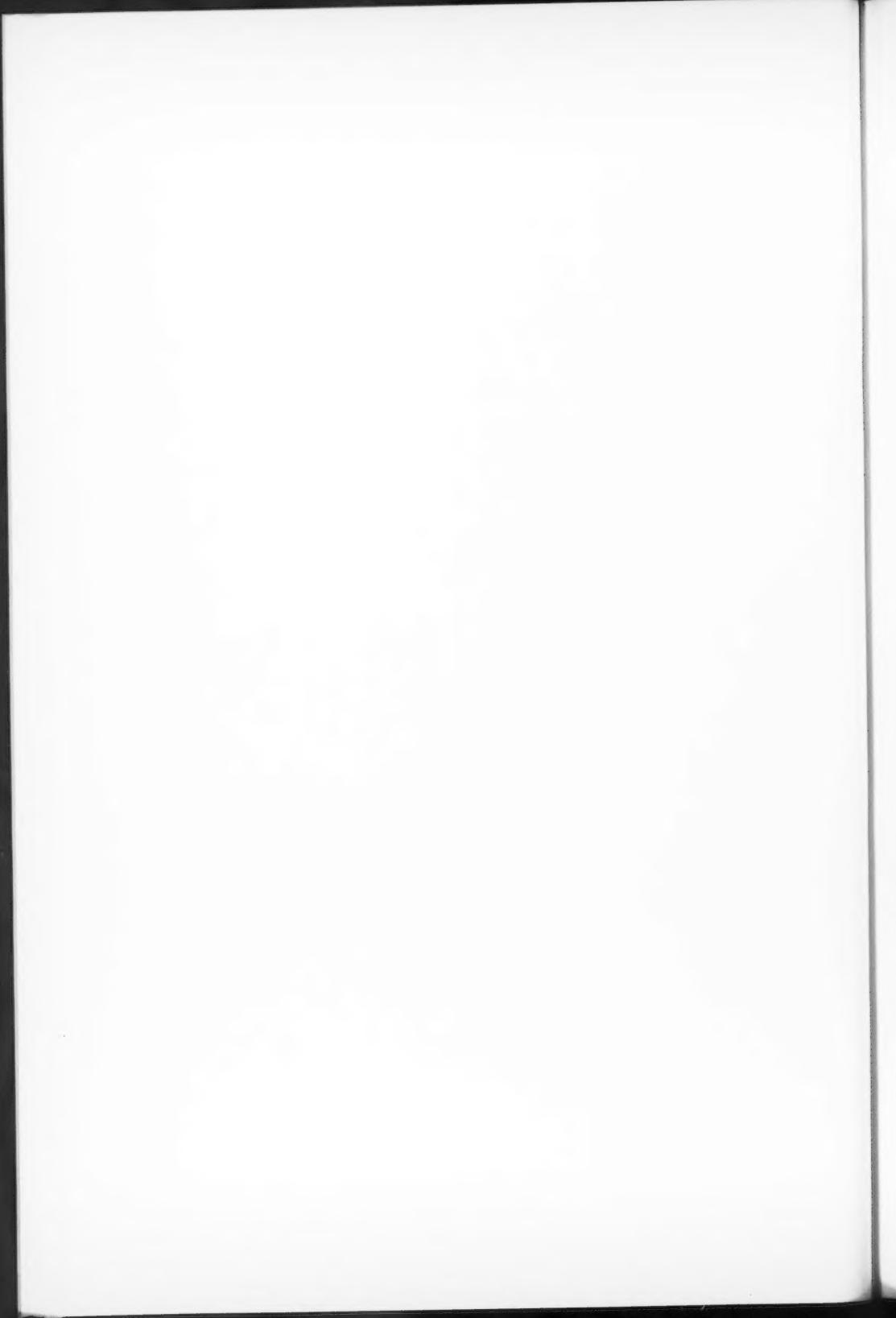
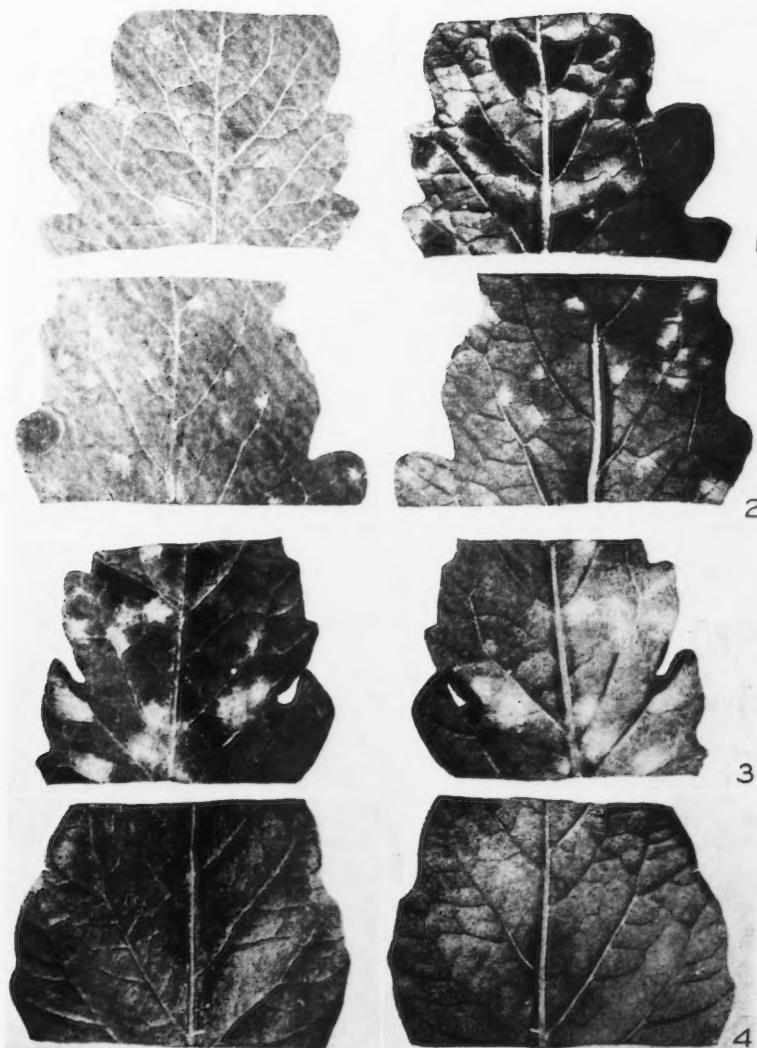
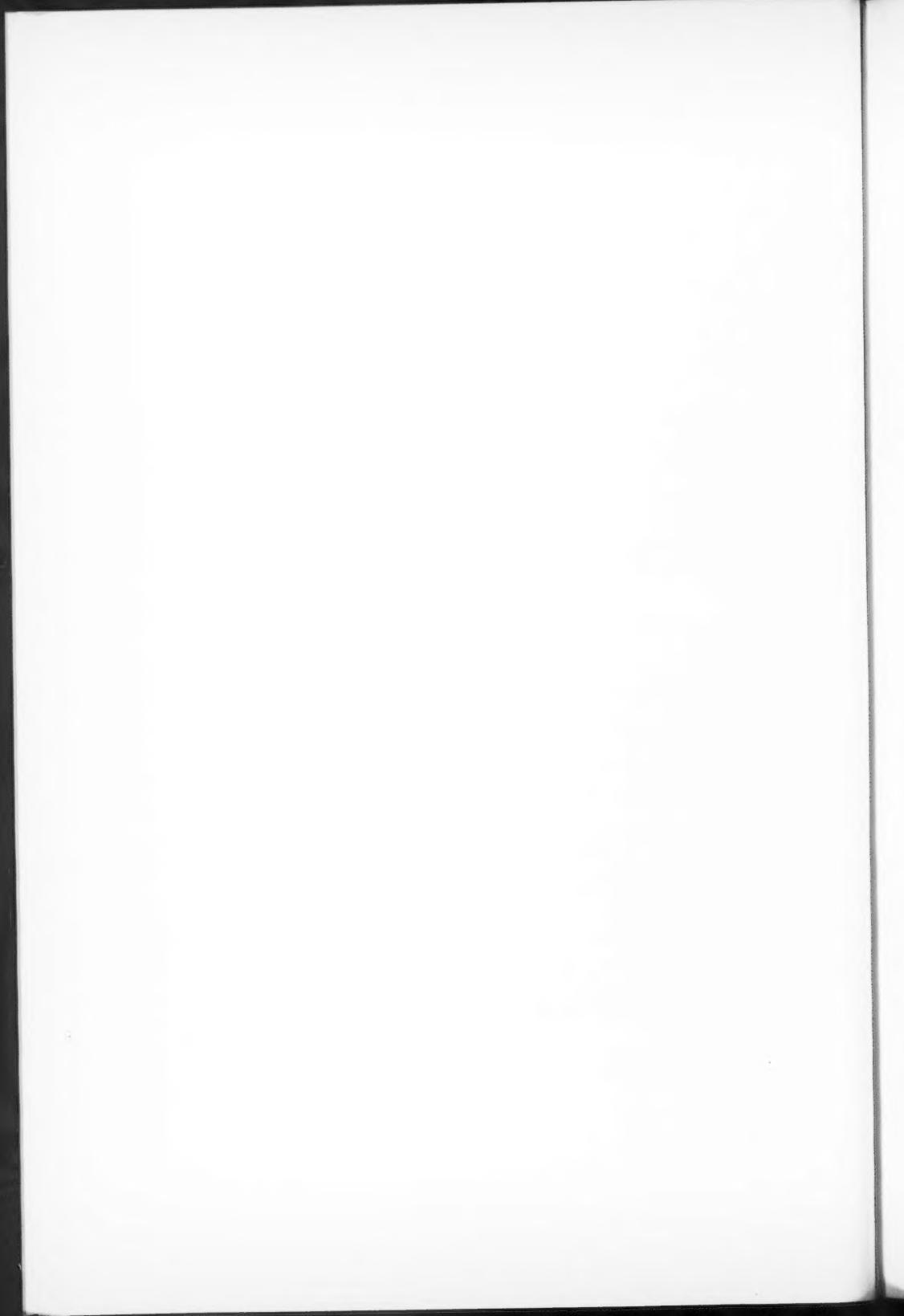


PLATE III



Figs. 1 TO 4. Reaction of vigorous mature plants of Potentate (Fig. 1), Stirling Castle (Fig. 2.), 2R Selection (Fig. 3.) and Red Currant (Fig. 4.) to *C. fulvum* physiologic Form 1, following a natural infection in August, 1935. Upper and lower leaf surfaces.  $\times 1$ .



fusion, should further resistance factors for this or other diseases be discovered in the tomato:

$Cf_{p1}$ —for the factor governing immunity.

$Cf_{p2}$ —for the factor governing partial resistance to all known forms of the fungus.

$Cf_{sc}$ —for the factor governing partial resistance to Forms 1 and 3, but not conferring resistance to Forms 2 and 4.

In these symbols  $Cf$  signifies a resistance to *C. fulvum* whereas the subscript indicates where the factor was first discovered: thus,  $p$  stands for *pimpinellifolium*, the species name of the Red Currant tomato and 1 and 2 signify the first and second resistances discovered in this species;  $sc$  refers to the *esculentum* variety Stirling Castle. It is hoped that other workers will employ this system of symbols if other resistance factors are discovered in the tomato. All three resistance factors have been located in MacArthur's (12) chromosome maps of the tomato. Such precise mapping of specific qualitative factors for disease resistance is probably unique in plants, although Abegg and Owen (1) have reported an instance of linkage between a factor for resistance to curly-top and a factor governing crown color in beets.

### Acknowledgments

I wish to express my gratitude for the assistance rendered by Professors D. L. Bailey, and J. W. MacArthur, under whose joint direction this investigation was carried out, and to Professor MacArthur for providing certain genetic materials. My thanks are also due to Professor E. F. Palmer for his co-operation in placing at my disposal the facilities of the Horticultural Experiment Station, Vineland Station, Ontario.

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## STUDIES ON THE GENUS *RHAGOLETIS* (TRYPETIDAE) WITH SPECIAL REFERENCE TO *RHAGOLETIS POMONELLA* (WALSH)<sup>1</sup>

By A. D. PICKETT<sup>2</sup>

### Abstract

This paper is a report on an investigation of the biological and morphological relations of a number of species of dipterous flies belonging to the genus *Rhagoletis* (Fam. Trypetidae). If the observations made and the conclusions drawn are valid, it would appear that the practice of separating species on the basis of very slight morphological differences, which may be due to host influences, is unsound.

The forms which develop in apple, hawthorn and blueberry, were studied extensively from the standpoint of their relations to their hosts and the conclusions reached are that one species *Rhagoletis pomonella* (Walsh) is involved and that the female insects show a decided preference to oviposit in the host in which they developed as larvae.

### Introduction

The dipterous insect *Rhagoletis pomonella* (Walsh), variously known as the apple maggot, railroad worm and blueberry maggot, is probably indigenous to eastern North America and has been mentioned in reports of entomologists and horticulturists for many years.

There has long been considerable doubt in the minds of entomologists as to the relationships of the forms found in the different hosts in which the insect breeds. Some authors have regarded the forms that breed in apple, hawthorn and blueberry as belonging to a single species, but of late there has been a tendency to regard the blueberry form as a distinct species and in 1932 (11) it was so described.

Judging from the literature on the subject it appears to be more or less generally accepted that the apple and hawthorn forms will develop in either host and that there are no definite biological or morphological differences between them. On the other hand attempts have been made by a number of investigators to show both biological and morphological differences between the apple and hawthorn forms on the one side and the blueberry form on the other. When the present investigation was undertaken, the writer was of the opinion that some differences actually existed.

In Nova Scotia, where these investigations have been carried on, the relationships of these forms are of considerable economic importance, since in the main fruit-producing area all three host plants are found growing in close proximity and consequently, if there is a free movement from one host plant

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to another the difficulty of controlling this pest on fruits of economic importance is increased. It was with the object of clearing up the relationships of these forms that these investigations were undertaken.

As the investigations progressed it occurred to the writer that the study of other closely related forms might be of some assistance in clarifying certain points, especially those dealing with the morphology of these forms. Consequently, specimens of the form found breeding in the snowberry in British Columbia were procured and studied. This form has been described as a distinct species under the name of *Rhagoletis symphoricarpi* Curran. It has not been possible to include this form in the biological investigations, but it would be desirable to do so were sufficient living material available. The forms found breeding in cherry offer very interesting material for study and it is regretted that breeding work with these could not have been included in the present investigation as this would appear to be a problem somewhat similar to that found in the case of *pomonella*.

While it has not been possible to carry out all the breeding work that appears necessary to clear up all the points in doubt, the writer is of the opinion that the results obtained to date indicate fairly clearly what the final outcome will be. It was originally intended to include genetical studies of the forms of *Rhagoletis pomonella*, but time has not permitted this, owing to the great amount of detail involved in the breeding work.

It is hoped that the present report will renew interest in this very interesting group of insects and that it may help to clear up what were, evidently, very erroneous ideas regarding the relationships of a number of forms belonging to this genus. The biological studies are being continued.

### General Considerations

In order to determine the relationships of the above mentioned group an investigational program was outlined. This was divided into two main sections: first, the morphology of the forms in question, and also of other related trypetids that were available; and second, a biological study of the forms developing in the apple, the hawthorn and the blueberry. The results of these investigations are given below.

### Morphological Studies

#### *Rhagoletis pomonella* (WALSH)

The *pomonella* group presents a difficult problem; and after a careful study of a long series of specimens, along with a painstaking survey of the literature, there is still considerable doubt as to the status of the various forms.

According to Illingworth (20), Porter (29) and Benjamin (4) the species *Rhagoletis pomonella* was described by Walsh (34) in 1867 under the genus *Trypeta*; the writer has not examined the original manuscript. This description refers to the flies developed on the apple and on the hawthorn; and there is no question as to its status. However, when Snow (31) described *zephyria* in 1894, the status of the group became somewhat complicated because the differences between the two forms were comparatively slight. Doane (12)

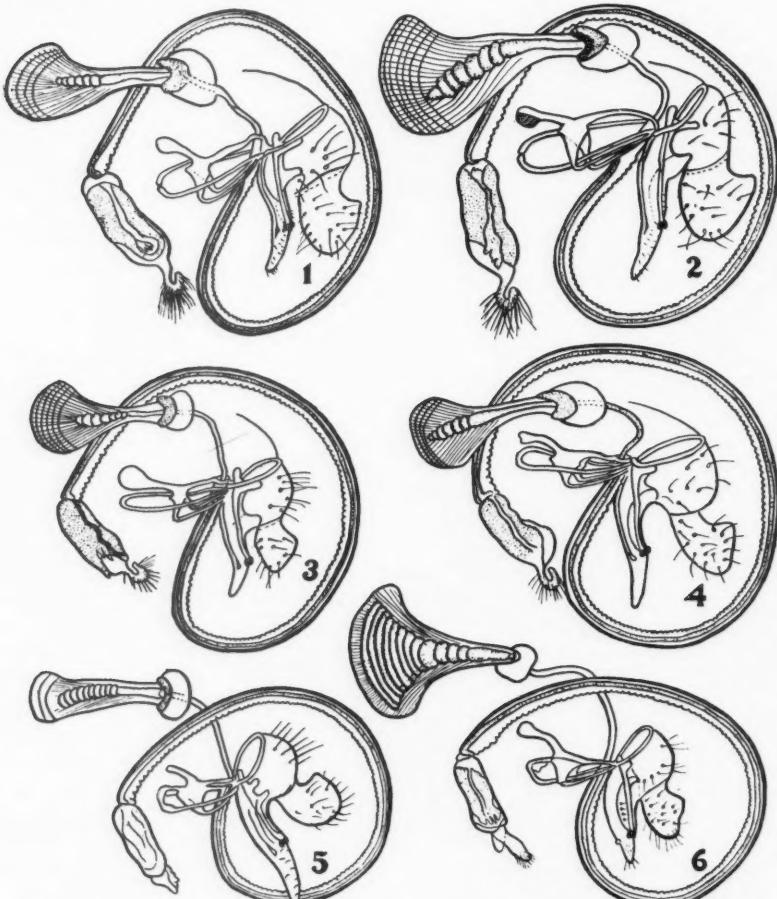
and Aldrich (1) questioned the validity of Snow's species. Curran (9, 10), however, after an examination of one female specimen accepted *zephyria* as a definite species on the basis of color variations between it and *pomonella* (Walsh). A related form, found in the snowberry *Symporicarpus racemosus* Michx., was described by Curran (9, 10) under the name *symporicarpi*, on the basis of the characters of the male genitalia. Cresson (8) regards the latter as a synonym of *pomonella*. Benjamin (4) has examined one of the cotypes of *zephyria* Snow, and cannot distinguish between it and those reared from the snowberry; consequently he has designated *symporicarpi* Curran as a synonym of *zephyria* Snow.

In 1932, Curran (11) examined specimens collected from blueberry, that were submitted to him by the writer for identification, and he described them as a new species *R. mendax*, on the basis of the characteristics of the male genitalia. Woods (35) studied the blueberry maggot in Maine, and after comparing all stages with Illingworth's (20) figures of *pomonella*, could observe no difference except in size and habits. In 1933 a series of specimens collected from apple, hawthorn, and blueberry were submitted to Dr. Alrich of the United States National Museum who, although he was unable to decide definitely (3), was of the opinion that there was not enough difference between the blueberry form and the others to give the former specific rank.

During the last four years the writer has spent a considerable amount of time studying the male genitalia of a number of species of *Rhagoletis*. A long series of the forms collected or reared from apple, hawthorn, blueberry and snowberry, have been carefully examined and a number of figures have been prepared.

There does not appear to be any constant difference in the male genitalia of the forms that occur on apple, hawthorn and blueberry, as a reference to Figs. 1, 2 and 3 will show. Curran's figures (11) are only partially complete; and the specimens were evidently not examined in the same plane. Further, his statements that the so-called sustentacular apodeme (ejaculatory apodeme) furnishes a "ready means of determining the species" and that "this organ exhibits striking differences in shape in the various forms studied" have not been substantiated by the writer's examination of a series in each group. In the specimens examined there is a great variation in the form of the ejaculatory apodeme in each group, and this character cannot be used for the purpose of identification. The writer is of the opinion, although this point was not definitely proved, that this organ develops and becomes larger in accordance with the period elapsing after the fly leaves the puparium. Specimens of apple and blueberry flies that had just emerged showed a weak development of this organ. A careful examination of the ejaculatory apodeme of each form shows that neither the crescentic rings, the width and shape of the apical portion, nor any other feature of this organ can be used to differentiate among these forms. A brief reference to Figs. 1, 2 and 3 will show some differences in the ejaculatory apodemes. These are drawings of specimens chosen at random for this purpose and none of the slight variations are constant. It will be noted that these figures show a greater difference in the size of this

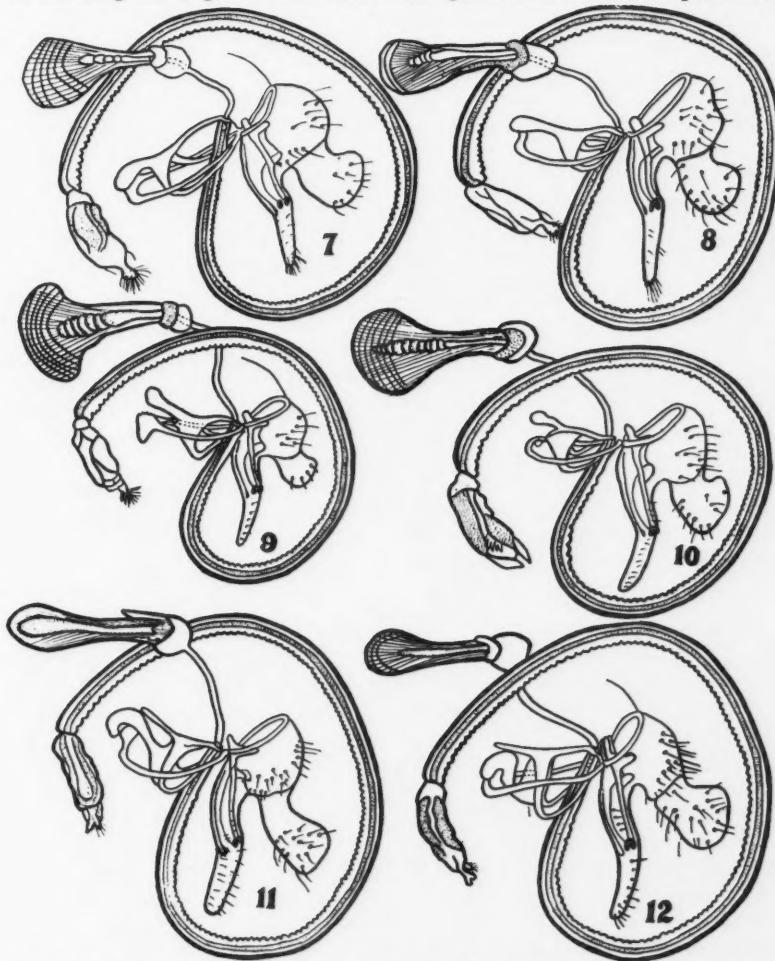
organ between the flies from hawthorn and those from apple than between the apple and blueberry forms; but the difference is not constant. The observations of the writer indicate that the tip of the penis is the most constant feature found in the male genitalia of all the species of the genus studied. As Curran (11) has pointed out, the proper orientation of these structures greatly affects the conclusions that may be drawn. If mounted in liquid glycerine these organs can be readily handled. The figures show the type of apical termination and, so far as can be ascertained from a study of a series of each form, the character is constant.



Figs. 1 to 6. All figures of male genitalia drawn to the same scale. FIG. 1. *Rhagoletis pomonella* (Walsh) (apple form). FIG. 2. *Rhagoletis pomonella* (Walsh) (hawthorn form). FIG. 3. *Rhagoletis pomonella* (Walsh) (blueberry form). FIG. 4. *Rhagoletis pomonella* (Walsh) (snowberry form). FIG. 5. *Rhagoletis tabellaria* (Fitch). FIG. 6. *Rhagoletis ribicola* Doane.

On a basis of the morphology of the genitalia of these three forms there are, in the opinion of the writer, no grounds for considering that more than one species is involved.

The figures shown by Benjamin (4) are different from those of Curran (10) in regard to the tip of the male clasper. The writer examined Snow's (31) figure of the wing of *zephyria* and came to the conclusion that the characters of the wing banding, on which Snow distinguished the form from *pomonella*,



FIGS. 7 to 12. All figures of male genitalia drawn to same scale. FIG. 7. *Rhagoletis cingulata* (Loew) (cultivated cherry). FIG. 8. *Rhagoletis cingulata* (Loew) (wild cherry). FIG. 9. *Rhagoletis berberis* Curran. FIG. 10. *Rhagoletis fausta* (Osten-Sacken). FIG. 11. *Rhagoletis suavis* (Loew). FIG. 12. *Rhagoletis completa* Cresson.

are not to be relied on. On the basis of an examination of ten specimens reared from each of the fruits, hawthorn, apple and blueberry, the hyaline indentation between crossbands two and three reach the fourth vein in some of each of these forms. In those bred on hawthorn, one specimen out of ten exhibited this condition; in the apple flies four specimens out of ten; and in the blueberry flies seven specimens out of ten showed this character.

The situation regarding the form from the snowberry is slightly different. The writer has not had an opportunity to examine the form reared from the sparkleberry *Batodendron arboreum* Marsh (Nutt) which Benjamin (4) considers to be conspecific with the snowberry form and also with Snow's *zephyria*.

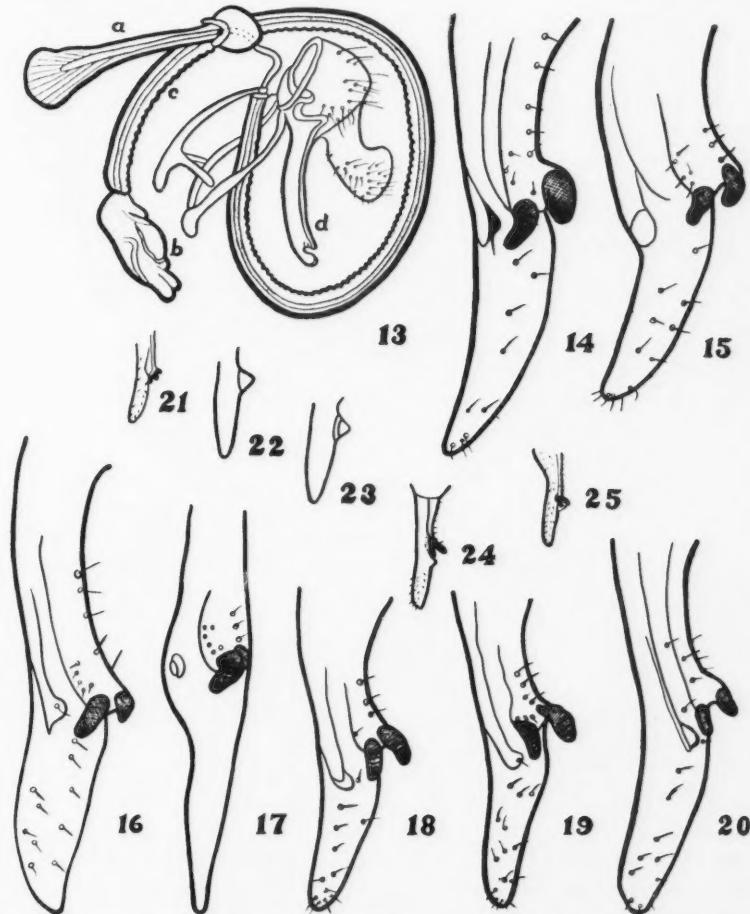
Curran (11), in reaffirming his identification of *symporicarpi* as a species, states that in his examination of the male genitalia he probably overlooked the most striking differences. Benjamin (5) maintains that the differences in the male genitalia as referred to by Curran (10) are sufficiently constant to separate this form from *pomonella* (Walsh).

The writer has examined the genitalia of a long series of each form and although certain characters mentioned by Curran (10) are fairly constant, even though somewhat variable, he cannot agree that the differences mentioned in Curran's (11) key are constant. Curran's (10) figures, however, are essentially correct in so far as they show the general shape of the claspers; but the statements regarding the lobe of the clasper and the length and shape of the hooks are not borne out by the present studies (Figs. 16-25). In most specimens, however, it is possible with careful orientation to see the more curved condition of the clasper in the apple form; the more ventral position of the apical tip or point of the clasper as compared with the form on the snowberry; and the wider and more compressed condition of the clasper beyond the spurs as seen in the latter. It is necessary to study these forms very carefully to note these apparent differences, as a slight variation in position during the examination entirely changes the appearance of these parts.

If one studied only the forms found on apple and snowberry it would be fairly easy to come to the conclusion that these differences were of sufficient significance and consistency to separate the forms as different species; but when the forms reared on hawthorn and blueberry are considered, some doubt develops. Some of the male hawthorn flies show an even greater divergence from those of the snowberry than do the apple flies. That is, the differences referred to above appear to be somewhat more intense (Figs. 14-16). This does not hold true for all individuals. When forms from the blueberry are studied, certain individuals, probably the minority, show these characters as being nearly midway between the apple and snowberry forms. These characters may be noted by comparing Figs. 14-25. With this additional information, the writer is inclined to believe that the snowberry form should not be regarded as a separate species.

One other feature which exhibits the very close relationship among all these forms is the nature of the tip of the penis, in which there is absolutely

no variation so far as can be found when complete specimens are examined. Among all the well defined species studied by the writer in this genus, with one possible exception, which will be referred to later, there were distinct differences in this part of this organ.



Figs. 13 to 25. FIG. 13. Male genitalia of *Epochra canadensis* Loew. a. Ejaculatory apodeme. b. penis. c. penis tube. d. claspers. FIGS. 14-25. Claspers of *Rhagoletis pomonella* (Walsh). FIG. 14. Right clasper of hawthorn form (latero-medial aspect). FIG. 15. Right clasper of apple form (latero-medial aspect). FIG. 16. Right clasper of snowberry form (latero-medial aspect). FIG. 17. Postero-lateral aspect of clasper shown in Fig. 16. FIGS. 18-20. Right claspers of blueberry form showing variations in apex. FIG. 21. Clasper of apple form showing three hooks. FIG. 22. Clasper of apple form showing shape of lobe opposite hooks. FIG. 23. Clasper of snowberry form showing shape of lobe opposite hooks. FIG. 24. Posterior aspect of clasper of apple form showing hooks and lobe. FIG. 25. Posterior aspect of clasper of snowberry form showing hooks and lobe.

A study of the larval characters which are mentioned by Greene (17) in his study of trypetid larvae was completed. This failed to indicate any significant differences between maggots taken from hawthorn, apple, blueberry and snowberry.

The specimens from snowberry were supplied by Mr. W. Downes, Victoria, B.C.

*Rhagoletis cingulata* (LOEW)

In the past this has been considered a very stable species with clearly defined characters, but in 1932 Curran (11) named two new species from closely related forms. The writer obtained specimens of both of these and dissected the male genitalia for comparison with the form reared from cultivated cherries. After comparing a series of the form which Curran (11) has designated as *indifferens* with a series of *cingulata* there is no doubt in the mind of the writer that the two are identical in so far as the male genitalia are concerned, and that *indifferens* should be considered as a synonym of *cingulata*. The differences mentioned by Curran (11) do not hold; and here again it may be pointed out that the size and shape of the ejaculatory apodeme is so variable in all these forms that it cannot be used as a taxonomic character. Reference to Figs. 7 and 8 will show that in the two forms figured, the form designated as *indifferens* by Curran has a somewhat wider ejaculatory apodeme than has the form from the cultivated cherry. This feature is not constant, however, and varies greatly in the specimens studied. The other differences mentioned, but not specified by Curran (11), were not observed by the writer. It will be noted also by the figures that the tip of the penis, which appears to be a fairly good taxonomic character, is similar in both forms. The findings as cited above agree with the observations of Benjamin (4) on forms reared in Florida.

The specimens of *cingulata* studied were supplied by Prof. L. Caesar, Guelph, Ontario, and Dr. F. L. Gambrell, Geneva, N.Y. The western form from the wild cherry was supplied by Mr. S. C. Jones, Corvallis, Oregon.

*Rhagoletis berberis* CURRAN

This is a new species established by Curran (11) on the basis of the nature of the wing banding. The wing pattern figured by Curran (11) appears constant in the specimens examined by the writer. These were from the type locality. Further, an examination of the male genitalia indicates that the species is distinct from *cingulata*. Figs. 7-9 show a number of differences, but the writer considers the nature of the tip of the penis to be of particular significance.

Curran (11) has mentioned the resemblances of this species to *R. completa* Cresson, and there does appear to be a fairly close relationship in both wing banding and male genitalia. The writer, however, is of the opinion that, as the differences found in the latter, as shown in the figures, are fairly constant and as the general body color is much darker in *berberis*, they should be regarded as distinct species.

Curran's types were reared from the fruits of *Berberis nervosa* at Hood River, Oregon, by Mr. S. C. Jones. The writer's specimens were supplied by Mr. Jones, but are labelled as being from grape.

*Rhagoletis fausta* (OSTEN-SACKEN)

There appears to have been very little confusion about the status of this species since Aldrich (2) established the synonymy of *R. intrudens* Aldrich. A good description of the species is given by Cresson (8) but the male genitalia have not been figured previously so far as can be ascertained. Fig. 10 shows that the male genitalia of this species are quite different from those of the other species studied, which indicates the value of these characters in a taxonomic study of the genus.

Specimens were obtained from Dr. F. L. Gambrell, Geneva, N.Y.

*Rhagoletis sauvis* (LOEW) AND *R. completa* CRESSON

*Rhagoletis sauvis* was described by Loew (22) in 1862; and in 1929 Cresson (8) established *completa* as a subspecies. Boyce (5) considers the differences great enough to establish the latter as a distinct species. He figures two characters found in the male genitalia, the validity of which the writer must question. It is true that there are slight differences in the claspers, but these are not as pronounced as shown by Boyce (5) and it would appear that a different perspective was used in each case. Boyce's figure of *completa* compares favorably with Fig. 12, but to get the claspers of *sauvis* to appear like those in Boyce's figure, it is necessary to orientate the part so that a postero-lateral view is obtained. Fig. 11 shows the condition observed by the writer. Further, these studies showed no constant difference in the size or shape of the ejaculatory apodeme, and as pointed out previously in connection with other species, this character cannot be relied upon in taxonomic determinations. A comparison of Figs. 11 and 12 shows these organs as they are found in the two specimens chosen at random for drawing. The distinguishing characters, as illustrated by Boyce (5) may occur in some specimens in either form, but cannot be considered as constant.

A reference to the figures will show the very close relation of the two forms. The differences between these are certainly not marked, the character of the wing pattern being probably the most pronounced. It was not possible to find any distinct differences in the tip of the penis. There may be sufficient justification for establishing *completa* as a subspecies, but in the opinion of the writer there is not sufficient evidence of differences to consider that two distinct species are involved. However, in order to establish definitely the status of those forms, genetical and cross-breeding investigations should be undertaken.

The specimens of *sauvis* were obtained from Dr. F. L. Gambrell, Geneva, New York, and those of *completa* from Dr. A. N. Boyce, Riverside, California.

*Rhagoletis tabellaria* (FITCH) AND *R. ribicola* DOANE

*Rhagoletis tabellaria* was described by Fitch (13) in 1856 under the genus *Tephritis*. In 1898, Doane (12) described a closely related form found on currant and gooseberry in Washington as *R. ribicola*. Aldrich (1) recognized this as a valid species and figured the wing. In 1915, Marcovitch (23) described a new species which he called *juniperinus* from flies reared on *Juniperus virginiana* in New York state. This was accepted by Phillips (27) as a valid species. The writer has not had an opportunity of studying the latter form, but the wing pattern as figured by Marcovitch (23) and Phillips (27) appears to resemble the form *ribicola* rather than *tabellaria*. Cresson (8) regards *ribicola* and *juniperinus* as synonyms of *tabellaria*. The writer has dissected and studied the male genitalia of one specimen of *ribicola* from Washington and considers it to be quite distinct from the specimen of *tabellaria* studied. Figs. 5 and 6 indicate very distinct differences between these species in regard to the shape and length of the claspers and also the nature of the tip of the penis. It will be noted that there are marked differences in the size and shape of the ejaculatory apodeme in the two figures, but it would require a study of a long series of specimens to determine the value of this character, and these are not available. However, there are such wide variations in these organs in all species where a series was available for study, that this character should be disregarded, at least until proved to be of value. On the basis of this examination the writer considers that *tabellaria* (Fitch) and *ribicola* Doane should both be regarded as distinct species.

Specimens examined were as follows: One specimen of *tabellaria* and one of *ribicola* from Mr. W. J. Brown, Entomological Branch, Ottawa; one specimen of *ribicola* from Mr. J. F. Gates Clarke, Pullman, Washington; one specimen of *tabellaria* from Dr. E. A. Chapin, United States National Museum, Washington, D.C.

**Biological Studies****HISTORICAL**

Walsh (34) described *Rhagoletis pomonella* from specimens reared from apple and hawthorn and therefore, inferentially, he regarded them as being identical. Other authors, so far as can be determined from the literature, have not considered there were differences between the flies that pass their larval stages in the apple and those found in haws.

In addition to the apple and hawthorn, crab apple, pear and plum have been reported as hosts; and Lathrop and Nickels (21) reared this species from the following plants found on blueberry barrens:

Blueberries: *Vaccinium angustifolium* Kalm (m.a.), *V. canadense* Ait. (m.a.), *V. corymbosum* L. (m.a.)

Bunchberry, *Cornus canadensis* L. (m.a.)

Chokeberry, *Aronia melanocarpa* (Michx.) Britton (m)

Huckleberry, *Gaylussacia baccata* (Wang) C. Koch (m.a.)  
Mountain cranberry, *Vaccinium vitisidaea* minus Lodd (m)  
Dwarf serviceberry, *Amelanchier bartramiana* Roem. (m)  
Wintergreen, *Gaultheria procumbens* L. (m)

(m) indicates fruits in which maggots were found, presumably *R. pomonella*.  
(a) indicates species from which adults were reared.

Whether these flies would change from one host to another has not been studied until recently, although several authors record observations on this point.

Illingsworth (20) published a detailed account of investigational work on *R. pomonella* carried on at Cornell University in 1911 and 1912. He also gives a technical description of all stages of the insect and records data on distribution and host plants. O'Kane (25) gave comprehensive historical and distributional reports, as well as information on the known hosts.

Woods (35) succeeded in successfully transferring very small larvae from huckleberries and blueberries to the fruit of the chokeberry, *Pyrus melanocarpa*, with the result that these individuals completed their development and pupated. He further observed that only in certain localized areas were blueberries infested with this insect, and that in many cases blueberries growing in the near vicinity of severely infested apple orchards showed no trace of the insect.

The same writer (35) failed to induce apple flies to oviposit in blueberries by confining them in cages on blueberries both in the field and laboratory. Attempts were made to transfer half-grown blueberry maggots to apples by inserting them beneath the skin; but these failed to develop. An attempt to induce flies taken on the blueberry to oviposit in apples also proved abortive. He does not state whether he succeeded in getting flies reared on any fruit to oviposit in captivity on that fruit. Wood's (35) conclusion is quoted as follows: "At any rate, the writer is inclined very strongly to believe that biologically at least there are two distinct strains or races of *Rhagoletis pomonella* Walsh, the one breeding in the apple and related fruits and the other in small fruits such as the blueberry and huckleberry. There does not seem to the writer to be any other conclusion which will explain the data given above. Certainly in so far as *Rhagoletis* occurs in Maine, the form on apple and the form on the blueberry are entirely independent." Similar observations and conclusions are recorded by Patch and Woods (26).

Porter (29) says, "Whether the flies which infest the different fruits are all of the same species is open to serious question. The occurrence of the species in fruit of hawthorn in localities in which the apple is free or virtually free from attack, the reverse condition in other localities, the presence of maggots in blueberries in certain restricted areas and in huckleberries in others, and the distinctly different habits of the blueberry flies from the flies in the apple orchard, all point to the possibility that there may be several

distinct species, biological races, or incipient species, which at present cannot be distinguished from one another."

According to Porter (29) the earlier investigators found difficulty in inducing flies to oviposit in cages.

Lathrop and Nickels (21) encountered difficulty in rearing specimens in confinement and, therefore, were unable to carry on cross breeding and other experiments to procure biological data on the relationship of the flies reared on different hosts. They were able to transfer first instar blueberry maggots to the apple and have them develop. From 200 second instar blueberry maggots treated as above, about 20 developed to the pupal stage and one adult was obtained. These were normal in size for the blueberry form. Second instar apple maggots were transferred to blueberries and some of these formed puparia but no adults emerged. The puparia were normal in size for the apple form. They also state that each maggot consumed two or more blueberries during its development. Their concluding paragraph is quoted, as follows: "It seems probable that the blueberry maggot and the apple maggot exhibit an example of incipient species formation, and from an ecological viewpoint the two forms seem distinct and independent."

In 1931, Fluke and Allen (14) reported on an investigation carried on in Wisconsin in which they secured ready mating and oviposition in cages by feeding a mixture of 1-3% yeast in 5% honey water. This greatly facilitated the carrying out of investigations on these insects.

McAlister and Anderson (24) working on the blueberry maggot in Maine used a modification of Fluke and Allen's method. They carried on inter-breeding experiments and found mating occurring when virgin females reared on apple were caged with males reared on the blueberry. However, copulation was less frequent when reciprocal crosses were made. No maggots developed from the latter cross, but of the ten apple females used in crosses with blueberry males, seven deposited eggs in blueberries from which 17 maggots hatched, 15 of which matured and pupated.

#### METHODS USED IN BREEDING WORK

During the first two years a fairly satisfactory technique for handling the flies was worked out. This is described as follows:

In the summer, heavily infested fields of blueberries were located and about the time the maggots began to emerge in numbers large quantities of berries were picked for the purpose of obtaining flies for the following season's investigations. The berries, in some cases, were spread out over the ground to a depth of one to two inches in a sheltered location and the maggots emerged from these and entered the soil to pupate. In other cases the berries were placed on soil in flats made with wire screen bottoms. These were set in the ground so that conditions would be as nearly normal as possible. When virgin flies were needed for crossing experiments, the flats were removed to the insectary the following June; but when they were used in studying host

relations, wire cages, constructed in a four-sided dome shape and covered with black cloth, were placed over the soil or flats in which the puparia were located. On the top of the cage was placed a small removable wire screen cage. The flies were allowed to enter this as they emerged from the soil, and a shutter made of a thin piece of board or shingle was arranged so that the passageway between the large and small cages could be closed. This allowed for the removal of the small wire cage so that the flies could be taken from it, or so that it could be replaced by another without the loss of the flies.

In the rearing of apple and hawthorn flies, heavily infested fruit was located in the autumn. When the majority of the maggots were nearing maturity the fruit was gathered and taken to the insectary. Here it was placed in boxes with coarse wire bottoms that would keep the fruit in, but would allow the maggots to pass through freely. Underneath these boxes were placed flats filled with ordinary fine garden soil. As soon as all the maggots had emerged from the fruit and entered the soil, the flats were set outdoors in the earth in a sheltered place, and left until the following June.

Just before it was time for the flies to emerge, the pupae were washed out of the soil by floating them in water. They were then put in sand, in wire cages made about 6 in. in diameter and 8 in. high with wooden tops and bottoms. These cages were covered with dark paper, and a  $\frac{3}{4}$  in. hole was made in the centre of the top of each cage. A paper cone leading up to this, inside the cage, directed the flies toward the opening. A glass tube connected this cage with a small wire cage about 3 in. by 3 in., constructed exactly as the larger ones, except that the wire screening was not covered. When the flies emerged they were attracted to the light, and consequently crawled up into the smaller cage. These small cages were made with a  $\frac{3}{8}$  in. glass tube in the top through which the flies might be fed. The flies were kept in the small cage until there were enough to take to the field, then the cage was removed and replaced by another.

Before any flies had emerged, clusters of fruits of susceptible varieties located in suitable places were selected, and breeding cages placed over them. The breeding cages were made of 20-mesh japanned wire screening. A circular piece of pine about  $\frac{3}{4}$  in. thick formed one end. In this two holes were bored, one  $\frac{3}{4}$  in. in diameter for putting the flies into the cage, and the other, through which the flies were fed, was  $\frac{3}{8}$  in. to hold a glass tube long enough to reach to the inside of the end and project about 1 in. on the outside. To the other end was attached a cotton sleeve. In apples and hawthorns, the end of the limb that bore the fruit to be covered was cut off just beyond the fruit, or in the case of a smaller limb, it was doubled back and the cage pulled up over the fruit and the cotton sleeve attached tightly to the limb above it. Usually another limb was tied to the first to prevent swaying and subsequent damage to the cage. The cages were placed so that they were fairly well protected and not exposed to too much direct sunlight. With the cages on blueberries great care had to be exercised in regard to the latter point. The cages were so close to the ground and the air moved through

them so slowly, owing to the protection afforded by the dense, low-growing vegetation, that a heavy mortality occurred during the bright warm days unless sufficient shade was provided.

When a sufficient number of flies were procured from the emergence cages, or from field collections, they were taken to the field cages in vials or in the small cages described above. In any case, the flies were put into the cage through the large hole in the end. The cages were connected by a piece of glass tubing about  $1\frac{1}{2}$  in. long and of the proper diameter to fit into the hole in the end of each. The small cage was then covered with black paper or cloth and the flies moved toward the light, to enter the larger cage. When they were all in, the small cage was removed and the opening in the breeding cage stoppered. The stopper could be easily removed to permit the addition of more flies. Although this method of transferring flies to the field cages was fairly satisfactory, it proved very slow at times, and in many cases the cotton sleeve was loosened and the small cage placed inside and left until feeding time the following day.

During the first two years of the investigations the flies were fed according to Fluke and Allen's (14) formula. In 1933, however, the writer was informed by Mr. James Marshall, Assistant Entomologist, State College of Agriculture, Washington, that Dr. L. B. Ripley (30), working on the Natal fruit fly in South Africa, had used cow's milk along with the Fluke and Allen mixture and had obtained excellent results. Dr. Ripley's letter to Mr. Marshall is quoted in part as follows: "The Fluke-Allen food does not develop eggs in our females and mating very rarely occurs on this diet, but when it is made with 50% milk, egg production is much increased and mating fairly frequent in large cages."

As this information was originally given to the writer verbally, he was under the impression that the percentage of milk was 5% instead of the 50% quoted above. However, the 5% mixture was used with fairly satisfactory results, although the stronger mixture might have been better. The mixture used was 5% honey, 5% milk and 2 to 3% yeast in distilled water. The mixture was made up fresh before each feeding and the feeding was ordinarily done quite early in the day, usually between eight and ten o'clock, as it was observed that the flies appeared to feed more at that time. Small pieces of absorbent cotton soaked in the feeding mixture were inserted into the glass tubes in the ends of the cages. New cotton was used at each feeding, that used the previous day being removed and discarded.

In securing virgin flies for cross-mating experiments, the methods described above were followed until the time the pupae were washed from the soil. In order to isolate each individual,  $\frac{5}{8}$  in. holes were bored in pieces of 2-in. planed hardwood planks about 4 ft. long. The holes were placed about  $\frac{1}{2}$  in. apart each way so that there were several hundred in each plank. Two or three thicknesses of blotting paper, cut to fit, were placed in the bottom of each hole. The pupae were placed one in each cell and a piece of fine wire screening was placed over the top of each and held in place by two tacks.

The planks were covered with damp newspapers to hold the moisture and then stacked up in the insectary. Each morning all flies that had emerged during the previous 24 hr. were removed and the cells containing pupae were moistened by a few drops of distilled water inserted with a medicine dropper. As the flies were removed they were examined and sorted according to sex and host plants and then placed in small wire cages like those described above, and fed. They were kept in the insectary until a sufficient number of each group had been accumulated to start a cage, when they were transferred to the field cages.

In the cross-breeding experiments the females were put in cages on the host on which they developed as larvae. It was thought that if mating did occur the females would be more likely to oviposit in these than in a fruit of some other species. Where males for the crosses were not available from reared material, they were collected from their host plants.

Observations were made each day on the flies in the cages and records kept of any data of interest such as the behavior, copulation, egg-laying, and so forth.

When the flies were all dead, or when there was evidence that the fruit was likely to deteriorate rapidly, the cages were removed and the fruit either deposited over earth in wire cages, or placed in glass vials covered with cheesecloth. When all the maggots had left the fruit, the earth was sifted and the pupae counted. Later they were placed in earth and put in a protected place out of doors for observation the following year.

The variety of apple used in the 1934 and 1935 investigations was the Bough Sweet. This is a soft, early, sweet variety, which is very susceptible to apple maggot attacks. Gravenstein was also used in the earlier investigations, but this variety is not as susceptible as Bough Sweet.

#### BREEDING INVESTIGATIONS (1931-32-33)

At the time these investigations were initiated there was no definite information regarding the biological relation of the flies developing on the apple, hawthorn and blueberry. In 1931, experiments were undertaken to find out whether the flies which had developed during their larval period in the fruits of one host could be induced to oviposit in another host, and if oviposition did take place, whether larvae would develop in the new hosts.

Since it was generally considered that the apple and hawthorn flies would cross from one host to the other fairly freely, the first experiments were to ascertain whether the blueberry fly would oviposit and develop in apple; this being the more important economic problem in Nova Scotia.

The breeding work undertaken may be divided into two divisions: First, to find out whether flies reared on one host would oviposit on another; and second, to determine whether flies reared on different hosts would interbreed.

## TRANSFERRING FROM ONE HOST TO ANOTHER

At Morristown, in 1931, a small Cox Orange apple tree, a variety susceptible to apple maggot attack, was entirely covered with a cheesecloth cage made as tight as possible. It was felt that if a large cage were used the flies would be more contented and more likely to oviposit. A large number of flies, approximately one hundred, consisting of both sexes, were gathered from blueberry bushes in an adjacent pasture and liberated in the cage. No attempt was made to feed these flies, as it was thought they would be able to find sufficient food in such a large cage (approximately  $12 \times 12 \times 10$  ft.). Although frequent examinations of the tree were made, none of the flies were ever observed and it was assumed that they either escaped or died. On trees nearby, blueberry flies were confined in cages similar to those described by Fluke and Allen (14) and fed on the yeast-honey-water mixture suggested by these workers. The flies were fed daily and lived on the average from ten days to two weeks. None was ever observed to make any attempt to oviposit and the flies seemed restless in the cages. When the cages were removed no stings were visible on the apples and no maggots were found in the fruits.

In 1932, more extensive trials were carried out. Before any flies emerged, cheesecloth bags were placed over fruits of hawthorns, blueberries, and susceptible varieties of apples. When flies began to appear on these fruits in locations where they were plentiful, they were captured in fairly large numbers and placed in cages on the various hosts mentioned; that is, flies collected from each host plant were confined in cages placed on apple, hawthorn and blueberry. During this year difficulty was encountered in keeping the flies alive for any length of time. Some difficulty was experienced at first in obtaining wire netting fine enough to hold the smaller flies collected from the blueberry, the wire used first being ordinary 14-mesh japanned wire screening. When 20 mesh copper wire was used, the flies did not appear to live a normal period and it was thought that the copper must be toxic to them. Finally, a suitable type of wire was procured, but it was then too late in the season for it to be of value. Difficulty was also experienced, especially in the cages on blueberry, in supplying the proper amount of shade. If the cages were too much exposed to the sun, a very warm day with bright sunshine would produce a high mortality. Similar observations have been reported by McAlister and Anderson (24). However, in spite of the difficulties encountered, the flies in cages on the host in which they developed as larvae appeared quite contented. Oviposition occurred, maggots developed, and puparia were formed. In only one did any crossing occur and that was in the case of the apple fly on hawthorn; and in one of these cages one maggot developed and formed a puparium.

The work carried on in 1933 showed, largely, a repetition of the 1932 results. The flies behaved normally in cages on the host plants in which they developed as larvae but no crossing occurred, and it appeared that if crossing did occur, it was so rare that it might be regarded as accidental. However, the economic importance of the problem was so great that it was decided to carry on the work another year.

## RESULTS OF BREEDING WORK (1934-35)

Table I shows the results obtained in the 1934 investigations. It is to be regretted that the data recorded were lacking in some respects, but it might be said also that data of uncertain validity were discarded.

It will be noted that eight of the 12 cages gave negative results. Cage 1 contained flies collected in the adult stage from the leaves of the apple. These flies seemed to behave normally and the apples were heavily marked by egg-laying punctures. It will be noted that 77 pupae were obtained.

Cage 2 contained flies that had developed as larvae in apples and had emerged from puparia in the insectary and were then transferred to the orchard. Why there was no oviposition here cannot be explained. It might be contended that these flies which emerged in the insectary were not as virile as those collected from the trees, but other data do not substantiate this, as the female apple flies used in Cage 3 also emerged in the insectary. In Cage 3 were apple females and blueberry males, and 27 pupae were obtained. This confirms the results of the work conducted in Maine by McAlister and Anderson (24). These flies appeared to behave in a normal way and lived a fairly long time, mating being noted on several occasions. No results were obtained in cages where blueberry or hawthorn flies were placed on the apple.

Blueberry flies collected from the bushes oviposited in Cage 8, and 21 pupae were obtained. Blueberry flies that had emerged in the insectary showed negative results in Cage 9, but the number of flies involved here was small. In Cage 12, apple flies collected from the trees oviposited in blueberries, and six pupae were obtained. The flies in this cage appeared quite contented after being caged for a few days and they were frequently seen feeding on the surface of the berries. The number of pupae collected appeared small compared to the number of berries apparently infested; it is felt that a number of larvae escaped before pupation, as three were found outside the cage. The report of McAlister and Anderson (24) is hereby confirmed, since it was the female apple flies which they reported as ovipositing in blueberries.

There is some doubt in regard to the results of the work on hawthorn, since the fruits were not covered early enough to preclude all possibilities of their being infested. They may therefore be disregarded.

No adults emerged from the cross-bred puparia that were kept over the winter, but this is not regarded as significant, as those reared on their own hosts also failed to emerge. It is thought that environmental conditions during the winter may not have been normal.

Table II shows the results obtained in 1935. It will be seen that information on Cages 28 and 29 is missing; this is due to the unreliability of the data. Some points of interest are as follows: In Cage 10, on blueberry, apple flies collected from the trees gave one pupa. Similar flies on blueberries in Cage 11 produced two pupae. All the crosses on blueberry gave negative results, and flies reared on the hawthorn also failed to develop on blueberry.

TABLE I

Cage No.	Host	Host in which reared		Place of emergence	No. of flies	Noted		Date started	No. of pupae
		Females	Males			Matting	Stings		
1	Apple	Apple	Apple	Apple trees	—	—	Yes	Aug. 9	77
2	Apple	Apple	Apple	Insectary	—	—	No	—	0
3	Apple	Apple	Blueberry	Insectary (females)	—	Yes	Yes	—	27
4	Apple	Apple	Hawthorn	Insectary (females)	Small	—	No	—	0
5	Apple	Blueberry	Blueberry	Collecting cages	—	—	—	—	0
6	Apple	Blueberry	Blueberry	Bushes	—	—	No	—	0
7	Apple	Hawthorn	Hawthorn	Bushes	—	—	No	—	0
8	Blueberry	Blueberry	Blueberry	Bushes	24	—	—	—	21
9	Blueberry	Blueberry	Blueberry	Insectary	Small	—	No	—	0
10	Blueberry	Blueberry	Apple	Insectary	5	—	No	—	0
11	Blueberry	Hawthorn	Hawthorn	Bushes	—	Yes	No	—	0
12	Blueberry	Apple	Apple	Apple trees	—	—	—	Aug. 9	6

TABLE II

Cage No.	Host	Host in which reared		Place of emergence	No. of flies	Noted		Date removed	Date started	Mating	Stings	No. of pupae
		Females	Males			No.	Yes					
1	Blueberry	Blueberry	Blueberry	Collecting cages	92	Yes	No	July 15	Aug. 16	—	—	0
4 and 4a	Blueberry	Apple	Apple	Insectary	200	No	Yes	July 20	Aug. 20	—	—	0
7	Blueberry	Blueberry	Blueberry	Collecting cages	62	Yes	No	July 20	Aug. 19	—	—	0
8	Blueberry	Blueberry	Blueberry	Collecting cages	49	—	—	July 22	Aug. 19	—	—	11
10	Blueberry	Apple	Apple	Apple trees	35	—	—	July 24	Aug. 19	—	—	1
11	Blueberry	Apple	Apple	Apple trees	44	—	—	July 24	Aug. 19	—	—	2
13	Blueberry	Hawthorn	Hawthorn	Insectary	114	—	—	July 26	Aug. —	—	—	0
16	Blueberry	Blueberry	Hawthorn	Insectary	5	—	—	Aug. 1	Aug. 19	—	—	0
17	Blueberry	Hawthorn	Hawthorn	Insectary	16	—	—	Aug. 1	Aug. 19	—	—	0
31	Blueberry	Hawthorn	Hawthorn	Bushes	80	—	—	Aug. 2	Aug. 24	—	—	0
35	Blueberry	Blueberry	Blueberry	Bushes	34	—	—	Aug. 13	Aug. 20	—	—	0
2	Apple	Blueberry	Blueberry	Collecting cages	199	Yes	Yes	July 18	Aug. 20	—	—	0
3	Apple	Blueberry	Blueberry	Insectary	181	Yes	Yes	July 20	Sept. 4	—	—	0
5	Apple	Apple	Blueberry	Insectary	122	Yes	Yes	July 20	Aug. 28	—	—	26
6	Apple	Apple	Apple	Insectary	170	Yes	Yes	July 20	Aug. 20	—	—	42
9	Apple	Apple	Apple	Apple trees	20	Yes	Yes	July 24	Aug. 20	—	—	16
12	Apple	Blueberry	Blueberry	Bushes	52	—	—	Aug. 28	Sept. 14	—	—	0
15	Apple	Apple	Hawthorn	Insectary	30	Yes	Yes	Aug. 25	Sept. 13	0	(1 dead larva)	0
33	Apple	Hawthorn	Hawthorn	Insectary	170	—	—	Aug. 5	Sept. 2	—	—	17
20	Hawthorn	Apple	Apple	Insectary	275	Yes	Yes	July 24	Sept. 13	—	—	17
21	Hawthorn	Apple	Apple	Apple trees	77	Yes	Yes	July 24	Sept. 13	—	—	9
22	Hawthorn	Apple	Blueberry	Collecting cages	114	Yes	Yes	July 25	Sept. 13	—	—	0
23	Hawthorn	Apple	Blueberry	Insectary and bushes	170	Yes	Yes	July 26	Sept. 13	—	—	0
24	Hawthorn	Apple	Apple	Insectary	160	Yes	Yes	July 29	Sept. 13	—	—	59
25	Hawthorn	Hawthorn	Apple	Insectary and apple trees	63	Yes	Yes	Sept. 1	Sept. 13	—	—	36
26	Hawthorn	Apple	Apple	Insectary	275	Yes	Yes	Sept. 2	Sept. 13	—	—	35
27	Hawthorn	Apple	Hawthorn	Insectary	180	Yes	Yes	Sept. 7	Sept. 13	—	—	0
14	Pear	Apple	Apple	Apple trees	55	—	—	Sept. 1	Sept. 13	—	—	3
18	Pear	Apple	Apple	Insectary	166	—	—	Sept. 1	Sept. 13	—	—	0
32	Pear	Apple	Apple	Insectary	140	Yes	Yes	Sept. 5	Sept. 13	—	—	0
19	Plum	Apple	Apple	Apple trees	235	—	—	Sept. 1	Sept. 13	—	—	0
30	Plum	Apple	Apple	Insectary	32	—	—	Sept. 1	Sept. 13	—	—	0
34	Chokeberry	Apple	Apple	Apple trees	140	No	Aug. 7	Sept. 30	Aug. Aug.	—	0	

In Cage 5 it will be noted that in the cross between apple females and blueberry males, positive results were obtained and 26 pupae were collected, which further confirms the work of the previous year.

Cage 12 is probably the most interesting of all. Here blueberry males and females collected from blueberry bushes produced 14 pupae in apples. So far as the writer can determine this is the first record of the blueberry form breeding in apple.

In Cage 15 a cross between apple females and hawthorn males on apple gave only one larva, which was found dead in an apple. In Cage 33 hawthorn flies which emerged in the insectary deposited eggs in apples, and 17 larvae developed and pupated.

Apple males and females appeared to behave in a normal way on the hawthorn, and pupae were procured from cages that contained flies that emerged in the insectary, as well as from those collected from apple leaves (Cages 20, 21, 24 and 26). Also, hawthorn females mated with apple males in Cage 25 produced offspring, and 36 pupae were procured.

The blueberry males and females on hawthorn in Cage 22 failed to produce pupae, although evidence of stinging was quite distinguishable and the flies appeared to be quite contented and lived a considerable length of time. An interesting observation made in connection with these flies was that they appeared to have difficulty in penetrating the haws to deposit eggs, and it may be that for this reason no maggots or pupae were found. A similar observation was made in connection with the blueberry flies on apple, although in one cage they did manage to deposit eggs. In Cage 23, containing hawthorn females and blueberry males, negative results were obtained.

Apple males and females were placed in cages on pear, plum and choke-cherry, *Prunus virginiana* L. In the last two, negative results were obtained, although the plum is recorded as a host by a number of authors, and the writer has taken larvae from an unknown variety of very early soft plum in Nova Scotia. In one of the cages on pear (No. 14), where flies collected from apple leaves were used, three pupae were obtained. This confirms the observations of Porter (29) and others that larvae of this species are occasionally found in pears.

One interesting observation made in these studies was in connection with the relative activity of the flies on the various hosts. Woods (35), Patch and Woods (26) and Caesar and Ross (6) point out that apple flies are much more sluggish than those found on the blueberry. This is true to a certain extent, but then again the hawthorn flies are more active than the apple flies, and the writer found them more difficult to collect than blueberry flies. It was noted that apple and hawthorn flies could be observed and captured much more easily on a bright, calm day, but on such a day it was difficult to observe blueberry flies, and it is thought that the direct rays of the sun, especially in the middle of the day, are too hot for them and they keep in the shade. They may be observed and captured more easily between eight

and ten o'clock in the morning and from four to six o'clock in the afternoon. The ideal kind of day to make observations on these flies, or to collect them, is a calm, dull, sultry day; and it was observed on more than one occasion that they could be taken with more than usual ease if an electrical storm was approaching.

It was found to be no more difficult to study blueberry flies in the field, when one had learned their habits, than apple flies. When one is looking down into a mass of vegetation it is more difficult to make observations, as the contrasts are much less sharp, than when examining leaves which are somewhat removed from other vegetation, as in the apple.

### Discussion of Results and Conclusions

It is difficult to draw definite conclusions from the data obtained, since some of them are conflicting and not entirely clear cut. However, as pointed out by Porter (29), negative results in breeding work are of no significance; since none of these flies were reared successfully in captivity until Fluke and Allen (14) worked out a satisfactory feeding mixture. The writer did not get positive results in crossing from one host to another until milk was added to the diet, and one cannot predict what may happen with further work on diet and improved methods of handling and breeding.

The writer has made many field observations regarding the habits of these flies, that are not enumerated above, but which have been reported on by other authors. When one observes hawthorns absolutely free from maggots year after year growing within a few feet of apples or blueberries that are very heavily infested, and *vice versa*, it is easy to see how the conclusion may be reached that there are some fundamental differences among these flies. However, since it has been possible in some cases, by using improved methods of feeding, to induce them to adopt hosts other than those on which they were reared, may it not be possible to obtain even more striking results if the proper environmental and dietary conditions are found?

*Rhagoletis pomonella* (Walsh) has generally been considered as an insect with food habits approaching the monophagous condition, and various authors have referred to it as having "strains" or "forms" feeding on and developing in certain specific fruits.

Some writers, particularly Curran (9-11), regard the forms feeding in the blueberry and the snowberry as distinct species. After spending considerable time studying flies and larvae from the different fruits, the writer is of the opinion that only one distinct species is involved, and that there is not sufficient evidence on the basis of biological or morphological interpretations, or both, to prove the existence of more than a single species. It would appear that a reasonable interpretation would be to regard *Rhagoletis pomonella* (Walsh) as an oligophagous insect, in which the "host selection principle", as enunciated by Hopkins (19), has become highly developed. This theory is quoted from Hopkins (19) as follows: "That an insect species which breeds

in two or more hosts will prefer to continue to breed in the host to which it has become adapted." One of the first entomologists to record observations in this connection was Walsh (33) who described the species now under consideration. However, numerous experiments which indicate that this phenomenon is not of rare occurrence in insects have been reported. Craighead (7) carried on investigations for six years with certain cerambycid beetles in the United States. Two of his conclusions that appear to have a bearing on the point under discussion are quoted here:

- (i) "In general, the fewer the hosts in nature, the more marked the pre-dilection for a particular host, and *vice versa*;"
- (ii) "Continued breeding in a given host intensified the preference for that host."

The work of Glendenning (16) in British Columbia on the satin moth, the observations by Fryer (15) in connection with apple capsids, the work of Thorpe (32) on *Hyponomeuta padella* L. in England, and of Pictet (28) in France, and of others, lend weight to this theory. Until such time as further investigations have disproved this hypothesis, it would appear to the writer a reasonable one to accept.

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